CHARACTERIZATION OF RAT COPPER/ZINC SUPEROXIDE DISMUTASE GENE AND CHROMATIN STRUCTURE ANALYSIS OF RAT MANGANESE SUPEROXIDE DISMUTASE GENE

By

JAN-LING HSU

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1993

TABLE OF CONTENTS

LIST OF FIGURES iii

ABBREVIATIONS v

ABSTRACT vi

CHAPTER 1 INTRODUCTION 1
Free Radicals and Superoxide Dismutases 1
Inflammatory Responses 5
Regulation of MnSOD by LPS, IL-1, and TNF 13

CHAPTER 2 MATERIALS AND METHODS 16
Materials 16
Methods 17

CHAPTER 3 RAT COPPER/ZINC SUPEROXIDE DISMUTASE GENE: ISOLATION, CHARACTERIZATION, AND SPECIES COMPARISON 26
Introduction 26
Results and Discussion 28

CHAPTER 4 CHROMATIN STRUCTURE ANALYSIS OF RAT MANGANESE SUPEROXIDE DISMUTASE GENE 43
Introduction 43
Results and Discussion 47

CHAPTER 5 HIGH RESOLUTION CHROMATIN STRUCTURE ANALYSIS OF RAT MANGANESE SUPEROXIDE DISMUTASE GENE 74
Introduction 74
Results 76
Discussion 92

CHAPTER 6 CONCLUSIONS AND FURTHER DIRECTIONS 98

REFERENCES 104

BIOGRAPHICAL SKETCH 117

LIST OF FIGURES

Figure		Page
3-1	The nucleotide and deduced amino acid sequences of rat Cu/ZnSOD cDNA	30
3-2	Tissue distribution of rat Cu/ZnSOD mRNA	31
3-3	Structure and restriction map of the rat Cu/ZnSOD gene	32
3-4	Southern analysis	34
3-5	Nucleotide and amino acid sequence of rat Cu/ZnSOD gene	36
3-6	Comparison of genomic organization of rat, mouse, and human Cu/ZnSOD genes	39
4-1	Transcriptional regulation of rat MnSOD gene by LPS	51
4-2	Genomic structure of rat MnSOD gene.	54
4-3	Probes design for DNase I hypersensitivity studies	56
4-4	Repetitive elements screening	57
4-5	DNase I hypersensitive sites in rat MnSOD gene (KpnI and probe PK)	59
4-6	DNase I hypersensitive sites in rat MnSOD gene (HindIII and probe KH)	61
4-7	DNase I hypersensitive sites in rat MnSOD gene (KpnI and probe KH)	64
4-8	DNase I hypersensitive sites in rat MnSOD gene (BamHI and probe BH)	67
4-9	DNase I hypersensitive sites in rat MnSOD gene (SspI and probe BH)	70

4-10 DNase I hypersensitive sites in rat MnSOD gene . . . 72

5-1 Protein-DNA interaction in rat MnSOD promoter region revealed by 4% polyacrylamide gel (LPS treatment) 78

5-2 Nascent RNA synthesis in permeabilized cells 81

5-3. Protein-DNA interaction in rat MnSOD promoter region revealed by 2.5% Metaphor agarose gel (LPS treatment) 83

5-4 Protein-DNA interaction in rat MnSOD promoter region revealed by 2.5% Metaphor agarose gel (IL-1 treatment) 86

5-5 Protein-DNA interaction in rat MnSOD promoter region revealed by 2.5% Metaphor agarose gel (TNF treatment) 88

5-6 The occupancy of basal and stimulated MnSOD promoter 91

ABBREVIATIONS

bp: base pairs

BSA: bovine serum albumin

Cu/ZnSOD: copper/zinc superoxide dismutase

DEPC: diethyl pyrocarbonate

DNase I: deoxyribonuclease I

HS site: hypersensitive site

IL-1: interleukin-1

Kb: kilobases

KD: kilodaltons

LPS: lipopolysaccharide

MnSOD: manganese superoxide dismutase

NF-κB: nuclear factor kappa B

PB site: protein-binding site

SDS: sodium dodecyl sulfate

SOD: superoxide dismutase

TNF: tumor necrosis factor

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF RAT COPPER/ZINC SUPEROXIDE DISMUTASE GENE AND CHROMATIN STRUCTURE ANALYSIS OF RAT MANGANESE SUPEROXIDE DISMUTASE GENE

By

JAN-LING HSU

December, 1993

Chairman: Dr. Harry S. Nick
Major Department: Biochemistry and Molecular Biology

A 13 kb rat copper/zinc superoxide dismutase (Cu/ZnSOD) genomic clone has been purified from a rat liver genomic library and completely characterized by restriction mapping, detailed sequencing and Southern blot analysis. This gene spans approximately 6 kb and contains five exons and four introns. Comparison of rat, mouse, and human Cu/ZnSOD genes reveals a high conservation in genomic organization and exon-intron junctions, including an unusual 5'GC donor sequence in the first intron. The gene contains a TATA box as well as an inverted CCAAT box, a feature common to both the mouse and human genes. Furthermore, several repeats were identified in the 5' promoter region of this gene, and these regulatory elements are also strikingly conserved in these three species.

In a separate study, I analyzed the molecular mechanisms controlling basal and stimulated rat manganese superoxide dismutase (MnSOD) expression. It has been previously demonstrated that the rat MnSOD, but not Cu/ZnSOD, mRNA levels were regulated by lipopolysaccharide, interleukin-1, and tumor necrosis factor. Nuclear run-on studies indicate that the increase in rat MnSOD mRNA level by these inflammatory mediators is in part due to transcriptional regulation. The regulatory mechanism has been further investigated by evaluating changes in MnSOD chromatin structure using Deoxyribonuclease I (DNase I) hypersensitivity studies. A total of seven hypersensitive (HS) sites have been identified along the rat MnSOD gene. The HS site identified in the 5' flanking region is highly sensitive to DNase I and following high resolution analysis was found to contain 4 discrete DNase I-resistant regions in control cells. It has been previously shown that DNase I-resistant regions are in fact protein footprints flanked by strong HS sites. More importantly, the cells treated with lipopolysaccharide, interleukin-1, and tumor necrosis factor contain an extra HS site in this 5' region, demonstrating that an additional transcriptional factor(s) involved in the regulation of rat MnSOD gene by these inflammatory mediators interacts with the promoter region.

CHAPTER 1
INTRODUCTION

Free Radicals and Superoxide Dismutases

A free radical is an atom or molecule that has an unpaired electron in the outer orbit. Free radicals, which occur in both organic and inorganic molecules, are sometimes generated in vivo as byproducts of normal metabolism. For instance, superoxide anion radical ($O_2^{-}\cdot$) is produced when oxygen is reduced to water by respiring cells. It is estimated that about 1-2% of O_2 consumed leaks form the mitochondrial electron transport system as $O_2^{-}\cdot$ (Boveris, 1977). The generation of $O_2^{-}\cdot$ in mitochondria are primarily from the sites of ubiquinone-cytochrome b (Boveris, 1977) and NADH-dehydrogenase (Turrens & Boveris, 1980). Free radicals can also be produced in response to bacterial invasion. In the site of infection, the neutrophils engulf and kill the invading microbes by the formation of oxygen-derived free radicals. In this case, superoxide and related radicals are generated by a tightly regulated, membrane-bound NADPH oxidase which is dormant in resting cells and becomes activated during phagocytosis (Bellavite, 1988).

Superoxide radical is very highly reactive and alone can cause considerable damage to cells. Neutralization of

the negative charge of $O_2^{-\cdot}$ by association with a proton, or with a metal cation, gives a stronger oxidant. These complexes can then initiate free radical chain reactions and substantially amplify the effects of the initiating $O_2^{-\cdot}$ (Fridovich, 1989).

The primary targets for cellular damage resulting from free radical formation are proteins, lipids, and DNA. Unsaturated and sulfhydryl-containing proteins are highly reactive with free radicals (Freeman & Crapo, 1982) and many enzymes can be inactivated by oxidation at their active sites. In addition, cytoplasmic and membrane proteins can be cross-linked into dimers or larger aggregates caused by free radicals damage. Lipid peroxidation occurs when free radicals react with the unsaturated bonds of membrane cholesterol and fatty acids (Svingen et al., 1978). The integrity and permeability of membranes can be undermined by the oxidation of membrane lipids and proteins. Furthermore, the nucleic acid base modifications and/or DNA strand scission caused by free radicals attack (Brown & Fridovich, 1981) can damage the genetic materials required for replication and transcription. Strong evidence has been presented indicating that many of the cell alterations seen in normal aging and in various diseases are due to oxidative damage from active oxygen species (Sinclair et al., 1990).

Cells are protected from free radical damage by a non-enzymatic and enzymatic antioxidant defense system. The

non-enzymatic antioxidants involved include compounds such as vitamin C and E. The enzymes involved in the antioxidant defense system are superoxide dismutases which dismutate superoxide anion radical into hydrogen peroxide and oxygen, catalases which convert hydrogen peroxide into water and oxygen, and peroxidases which reduce hydrogen peroxide into water.

Superoxide Dismutases

The superoxide dismutases (SODs) catalyze the dismutation of superoxide anion radical into hydrogen peroxide and oxygen as follows:

$$2H^{+} + 2O_2^{-\bullet} \rightarrow H_2O_2 + O_2$$

SODs, isolated from a wide range of organisms, fall into three types depending on the metals found in their active centers: copper/zinc, manganese, and iron. Cu/ZnSOD is found mainly in the cytosol of eukaryotes (Slot et al., 1986) and in chloroplasts; MnSOD, in prokaryotes and the mitochondria of eukaryotes; and FeSOD, in prokaryotes and in a few families of plants. In addition, an extracellular SOD similar to the Cu/ZnSOD has also been found in the extracellular fluid of eukaryotic cells (Marklund, 1984; Hjalmarsson et al., 1987). Amino acid sequence data has shown that these three types of SODs fall into two distinct families (Bannister et al., 1987). The FeSOD and MnSOD show a high degree of amino acid sequence and structural

homology, while the second family, which includes the Cu/ZnSOD, is completely unrelated.

Two major forms of SOD have been purified from rat liver (Asayama et al., 1985). The Cu/ZnSOD which has been found predominately in the cytosol (Slot et al., 1986) consists of two identical subunits with a total molecular weight of 32 kD. Each subunit contains a single Cu and Zn atom, noncovalently linked. The copper ion appears to function in the enzymatic reaction, whereas the zinc ion does not function in the catalytic activity but stabilizes the enzyme. The complete amino acid sequence of Cu/ZnSOD has been determined from various species with a high level of conservation (Bannister et al., 1987).

The rat MnSOD is a tetrameric enzyme containing four identical 21 kD subunits with a total molecular weight of approximately 80 kD whereas most bacterial MnSODs are dimeric enzymes of approximately 40 kD. This enzyme contains Mn at its active site and has been localized primarily in the mitochondria, particularly the matrix between the cristae (Slot et al., 1986). Mitochondrial DNA and membranes are particularly prone to oxidation because mitochondria themselves are a major source of free radicals. When mitochondria are severely damaged, aerobically growing cells are starved for energy. The localization of the MnSOD exclusively within the mitochondria suggests an important role for this antioxidant enzyme in this organelle. The

amino acid sequences of the MnSOD from various species, including rat, mouse, and human, as well as from bacteria, have been compared and are very similar between these species (Bannister et al., 1987).

Both rat Cu/ZnSOD and MnSOD cDNAs have been isolated and characterized by our laboratory and others (Ho & Crapo, 1987). The rat Cu/ZnSOD cDNA encodes a protein of 154 amino acids. The rat MnSOD cDNA encodes a protein of 222 amino acids which includes a putative mitochondrial targeting sequence of 24 amino acids. The Cu/ZnSOD is sensitive to cyanide, whereas MnSOD is resistant to this reagent (Weisinger & Fridovich, 1973; Fridovich, 1974). The difference in the cyanide sensitivity makes it possible to distinguish the enzymatic activities of these two SODs.

In general, the synthesis of Cu/ZnSOD is constitutive whereas MnSOD is inducible. Induction of MnSOD in eukaryotes has been observed following treatment with paraquat (Krall et al., 1988), X-irradiation (Oberley et al., 1987), and hyperoxia (Freeman et al., 1986), suggesting that MnSOD induction is important for protection against oxidative stress. Since inflammatory responses often result in generation of a variety of free radicals which can directly or indirectly lead to tissue injury, it is of interest to further characterize the role of SOD in such inflammatory responses.

Inflammatory Responses

Microbial invasions, immunological reactions, and inflammatory processes induce a complex set of responses in the host, collectively referred to as the acute phase response (Kushner et al., 1989). This response is characterized by fever, metabolic changes, increased peripheral white blood cell count, and an increased synthesis of acute phase proteins including C-reactive protein, ceruloplasmin, serum amyloid A protein, and various complement proteins.

In addition, the immune system produces a variety of inflammatory mediators including cytokines, arachidonic acid metabolites, and oxygen free radicals to protect the host when threatened by inflammatory agents, microbial invasion, or injury. In some cases this complex defense network successfully restores normal homeostasis, but at other times the overproduction of inflammatory mediators may actually prove deleterious to the host (Welbourn & Young, 1992). For example, septic shock associated with infection is generally caused by wide spread and uncontrolled activation of the mononuclear phagocyte cell population and the release of massive quantities of inflammatory mediators (Molloy et al., 1993). The clinical characteristics of this condition include fever, hypotension, hypoglycemia, disseminated intravascular coagulation and increased vascular permeability.

Lipopolysaccharide and its Receptors

One of the major toxic components which contribute to the inflammatory responses, as a result of microbial invasions, is lipopolysaccharide (LPS). LPS, which are cell wall constituents of gram-negative bacteria, consist of a polysaccharide portion and a covalently linked lipid moiety termed lipid A (Rietschel & Brade, 1992). Wide derangements which include acute inflammatory responses, altered energy metabolism, and multiple tissue injury are observed after LPS administration (Ghosh et al., 1993). The toxic properties of LPS account for its other name, bacterial endotoxin.

Several lipopolysaccharide binding proteins (LBP) which specifically interact with LPS have been reported (Tobias, 1988). Schumann et al. (1990) have characterized a LBP which is a 60 kD plasma glycoprotein synthesized in hepatocytes. After the LBP interacts with LPS, this LPS-LBP complex then binds to the cell surface protein CD14 and presumably elicits the cellular and tissue responses to LPS (Wright et al., 1990). In addition, a membrane-localized 80 kD LPS-specific binding protein has been identified in B lymphocytes, T lymphocytes, and macrophages (Lei & Morrison, 1988). However, the roles of these different LBPs and the interaction between them are still not clear.

The exact mechanism underlying the various forms of inflammation is complex, because it involves an extensive

network of interacting mediators belonging to various molecular categories. Cytokines, such as interleukins, tumor necrosis factor, and interferons, are now considered as the primary factors in the cascade of events that regulate the inflammatory response. Interleukin-1 (IL-1) and tumor necrosis factor(TNF) are two of the major cytokines induced by LPS, and are thought to play an important role in mediating the pathophysiological effects of LPS (Fenton et al., 1987; Baud et al., 1989). These two cytokines differ in structure, but have many of the same biological activities (Le & Vilcek, 1987; Larrick & Kunkel, 1988). These three inflammatory mediators, LPS, IL-1, and TNF, have each been shown to induce other gene products associated with the onset of inflammation such as neutrophil chemotactic factor (Strieter et al., 1989) and mitogenic proteins secreted by both melanoma and human endothelial cells.

Interleukin-1 and its Receptors

IL-1, initially discovered as a factor responsible for producing high fever, is primarily synthesized by macrophages and monocytes, but is also found in keratinocytes, glial cells and endothelial cells. Specific physiological activities of IL-1 which have been documented include lymphocyte stimulation, induction of acute phase proteins, helper activity for immunoglobulin production by B

lymphocytes, induction of interferon β-2, and radioprotection (Neta et al., 1986; Fibbe et al., 1989).

Two forms of IL-1 protein have been isolated: alpha and beta. These two proteins have been found in human, mouse, rat, pig,and rabbit and exhibit significant interspecies homology at the amino acid level (62% homology between human and mouse IL-1α). IL-1α and IL-1β are encoded by two distinct genes that each code for precursor polypeptides (Pro-IL-1α and Pro-IL-1β) of predicted molecular weight 31 kD (Auron et al., 1984; Lomedico et al., 1984; Clark et al., 1986; Dinarello, 1992). Both Pro-IL-1α and Pro-IL-1β undergo enzymatic cleavage, resulting in the 17 kD products of isoelectric point 5 and 7, respectively. IL-1α and IL-1β exhibit limited homology (26% in human), yet exert similar or identical biological effects and appear to interact with the same receptors.

Two types of IL-1 receptors have been isolated and characterized (Dower et al., 1992). The type I IL-1 receptor purified from a murine T-cell line is an 80 KD glycoprotein which is a member of the Immunoglobulin (Ig) superfamily (Sims et al., 1988). The extracellular IL-1 binding portion of the receptor comprises 391 amino acids, the cytoplasmic part is 217 amino acids long, and there is a presumed 21 amino acid transmembrane region. The type II IL-1 receptor has a molecular weight of about 68 KD and is also a member of the Ig superfamily. The two IL-1 receptors

show approximately 28% homology in their extracellular domains, but differ significantly in their cytoplasmic domains: only 29 amino acid residues for the type I receptor, in contrast to 213 amino acid residues for the type II receptor.

Receptor binding studies have indicated that IL-1β precursor protein requires processing to express receptor binding and biological activity, whereas the IL-1α precursor is capable of binding to the IL-1 receptor and hence is presumably biologically active (Mosley et al., 1987). Studies in fibroblasts and T-lymphocytes have demonstrated that IL-1 receptor is rapidly internalized and subsequently becomes localized in the nucleus (Perlmutter et al., 1986; Mizel et al., 1987). This internalization is associated with the down-regulation of IL-1 responsiveness and therefore may represent a feedback mechanism.

Tumor Necrosis Factor and its Receptors

TNF, also called cachectin, is synthesized principally by monocytes and macrophages in response to macrophage activators such as endotoxin. Human TNF has a molecular weight of 17 kD, which is synthesized as a propeptide of 233 amino acids with a precursor sequence of 76 amino acids (Pennica et al., 1984; Aggarwal et al., 1985). The amino acid sequences of both the pro-and mature peptides are highly conserved between mouse and human, with about 80% homology. A wide spectrum of possible physiological actions

for TNF, including its use in the management of certain tumors, its role in an inflammatory reaction, and its involvement in the immune response, have been documented (Beutler & Cerami, 1989).

TNF and lymphotoxin, sometimes referred to as TNFα and TNFβ respectively, are structurally and functionally related (Goeddel et al., 1986). They share a 28% homology, and have been shown to interact with the same receptors and exhibit a number of shared biological activities. The genes for TNF and lymphotoxin are closely linked and separated by about 1 kb in humans (Nedwin et al., 1985). Lymphotoxin lies 5′ to TNF, and both genes reside within the major histocompatibility complex locus on chromosome 6, indicating that these two genes may have evolved from a gene duplication event.

Most cell types including liver, kidney, muscle, and adipose tissue have been shown to possess specific high affinity receptors for TNF. Two distinct TNF receptors of 55-69 kD (p55) and 70-80 kD (p70) have been identified in the mouse (Goodwin et al., 1991) and human systems which demonstrate ligand specificity for both TNFα and TNFβ (Hohmann et al., 1990). The p70 receptor possesses greater affinity for TNF than the p55 receptor (Tartaglia & Goeddel, 1992). The genes for the p55 and p70 receptors map to chromosomes 12 and 1 in humans, and to chromosomes 6 and 4 in the mouse (Goodwin et al., 1991).

Based on their amino acid sequence, the two receptors are only 29% identical to each other, and both share four cysteine-rich repeats in their extracellular domains that are characteristic of the NGF receptor family. For both proteins, aggregation of receptors mediated by binding of dimeric or trimeric TNF is believed to initiate the signalling process. However, several lines of evidence suggest that subsequent steps of the signal transduction process may diverge. Studies have been conducted using antibodies specific for either the p55 or p70 receptor. Heller et al. (1992) reported that the cytotoxicity of TNF was mediated through the p70 TNF receptor. Tartaglia et al. (1993) provided evidences indicating the cytotoxicity of TNF is signaled by the p55 TNF receptor. The controversy between these two hypotheses needs further investigation, but it also demonstrates the complexity of the TNF function.

Soluble forms of the TNF receptors are released from the cell surface by proteolytic cleavage (Lantz et al., 1990; Gullberg et al., 1992). These soluble receptors act as TNF binding proteins, subsequently inhibit TNF interacting with surface receptors. In addition, the release of soluble receptors results in the down-regulation of cell surface receptors. Therefore, shedding of surface receptors enables target cells to decrease their responsiveness to TNF. Binding of TNF to its receptor is followed by rapid internalization and degradation via a

lysosomal pathway (Tsujimoto et al., 1985; Niitsu et al., 1985).

Regulation of MnSOD by LPS, IL-1, and TNF

LPS, IL-1, and TNF have been demonstrated to induce MnSOD at both the protein and mRNA level. It has been reported that LPS increased MnSOD protein level in human monocytes (Asayama et al., 1985a) and bovine pulmonary endothelial cells (Shiki et al., 1987). TNF and IL-1 were also found to induce the mRNA for human MnSOD, but not the mRNAs for other antioxidant enzymes, such as catalase and glutathione peroxidase (Wong & Goeddel, 1988). Interestingly, the mRNA and protein level of Cu/ZnSOD is not changed following the treatment of LPS, IL-1 or TNF.

While the detailed mechanisms of how and why LPS, IL-1, and TNF regulate MnSOD expression are still unclear, the involvement of superoxide production is suspected. LPS, IL-1, and TNF have been shown to stimulate superoxide formation in mononuclear cells and endothelial cells (Matsubara & Ziff, 1986; Jones, 1986; Berkow et al., 1987). There is abundant evidence that $O_2^{-}\cdot$ generated and released during the respiratory burst is associated with not only host defense through microbial killing and tumoricidal activities, but also with tissue injury accompanying inflammatory reactions (Ward et al., 1988). Therefore, the elimination of superoxide radicals by MnSOD may provide an

alternative choice to protect the body against the tissue injury caused by the high concentration of $O_2^{-\cdot}$ induced by LPS, IL-1, and TNF.

Several lines of evidences indicate the protective role of MnSOD. When transgenic mice overexpressing human MnSOD are exposed to 95% oxygen, they showed less pathological damage and survived significantly longer than nontransgenic littermates, demonstrating the role of MnSOD in the protection from oxygen injury (Wispe et al., 1992). Eastgate et al. (1993) have shown that the increased radioprotection by IL-1 pretreatment correlates with the induction of MnSOD, indicating that MnSOD might play an important role in radioprotection.

Wong et al. (1989) characterized the role of MnSOD in protecting the cell against cytotoxicity of TNF by overexpressing MnSOD in TNF-sensitive cells. They reported that the expression of MnSOD reduced cellular sensitivity to TNF cytotoxicity, and expression of anti-sense MnSOD RNA resulted in cells which were sensitive to TNF. Therefore, they suggested that MnSOD was an important determinant of cellular resistance to TNF and argued that mitochondrially generated $O_2^{-\cdot}$ was a major component of TNF-mediated tumor cell killing.

Schulze-Osthoff et al. (1992) have further provided evidences to support the hypothesis that TNF cytotoxicity is mediated through the generation of reactive oxygen

intermediates. Their results have indicated that upon TNF stimulation mitochondrial radicals are formed at the ubiquinone site of the respiratory chain, where the electrons are directly transferred from ubisemiquinone to molecular oxygen, and this one-electron transfer results in the formation of superoxide free radicals.

In our laboratory, we found that MnSOD mRNA level in L2 cells, a normal rat pulmonary epithelial-like cell line, was elevated by LPS, IL-1, and TNF (Visner et al., 1989). It is, therefore, of interest to further characterize the regulatory mechanism of MnSOD expression by these three inflammatory mediators.

CHAPTER 2
MATERIALS AND METHODS

Materials

A rat liver cDNA library in lambda gt11 was purchased from Clontech Laboratories. A rat liver genomic library in lambda Charon 4A was kindly provided by Dr. Thomas Sargent (Sargent et al., 1979). The nitrocellulose filters used for cDNA and genomic libraries screening were obtained from Micron Separations Inc. The polyclonal antibody against rat Cu/ZnSOD protein was provided by Dr. Ian Burr (Asayama & Burr, 1985). Sequenase DNA sequencing kit was a product of United States Biochemical Corporation. Restriction endonucleases were mainly purchased from Bethesda Research Laboratories and New England Biolabs. The noncharged nylon membrane used for electrotransfer were obtained from Du Pont-New England Nuclear (GeneScreen) or Cuno, Inc. (Zetabind). L2 cell line and human Cu/ZnSOD cDNA were obtained from American Type Culture Collection. Ham's modified F12K medium was purchased from GIBCO, and fetal bovine serum from Flow Laboratories. Materials purchased from Sigma include bovine serum albumin, antibiotic antimycotic solution, *E. coli* LPS (*E. coli* serotype 055:B5), and lysolecithin. Recombinant human IL-1α and TNF were

obtained from the National Cancer Institute and Genentech Corp., respectively. Radioactive materials, [α-^{32}P]UTP (3000 Ci/mmol) and [α-^{32}P]d-ATP (3000 Ci/mmol), were ordered from Du Pont-New England Nuclear. DNase I used for hypersensitivity studies was purchased from Worthington. Random primer DNA labeling system for probe preparation and 100 bp DNA ladders were obtained from Bethesda Research Laboratories. Agarose and Metaphor agarose were purchased from FMC BioProducts.

Methods

Screening of cDNA and Genomic Libraries

A rat liver cDNA library in lambda gt11 was used to screen for rat Cu/ZnSOD cDNA clones. For immunoscreening (de Wet et al., 1984), the cDNA library was plated on *E. coli* Y1090, induced with IPTG, and transferred to nitrocellulose filters. These filters were incubated with polyclonal antibody against rat Cu/ZnSOD protein at 4°C for 16 hours, then treated with secondary antibody, a goat anti-rabbit antibody coupled to horseradish peroxidase. Positive clones were visualized with the chromogenic reagent, 4-chloro-1-naphthol. The positive clones obtained from immunoscreening were further confirmed by hybridization with a human Cu/ZnSOD cDNA probe.

For cDNA and genomic libraries screened using radiolabeled DNA probes, the phages were plated on *E. coli*

Y1088 and transferred to duplicate nitrocellulose filters. The filters were denatured in 1.5 M NaCl, 0.5 M NaOH, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0, washed in 1X SSC (0.15 M NaCl, 0.015 M Sodium citrate, pH 7.0), and baked under vacuum for 2 hours at 80°C (Maniatis et al., 1982). All the DNA probes used for library screening were radiolabeled by the random primer extension method (Feinberg & Volgelstein, 1983) and purified through a G-50 column. The filters were washed in a solution containing 0.05 M Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA and 0.1% SDS for 30 minutes and then transferred to a hybridization solution containing 0.05 M Tris-HCl, pH 7.5, 1 M NaCl, 10 mM EDTA, 0.1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll type 400, 0.2% BSA, and 100 μg/ml sheared, denatured salmon sperm DNA. The filters were hybridized with radioactive probe overnight and washed in a solution of 1X SSC and 0.1% SDS at the appropriate temperature. The temperature used for rat Cu/ZnSOD cDNA probe was 65°C and for the human Cu/ZnSOD cDNA probe, 55°C.

One of the purified rat Cu/ZnSOD cDNA clones, c14-2, was then used as a probe to screen a rat liver genomic library in lambda Charon 4A. The protocol for genomic library screening is similar to the protocol as described above for the cDNA library screening.

Subcloning and Sequencing

All the positive clones obtained from both cDNA and genomic library screening were subcloned into pUC19 using the EcoRI restriction site. In addition, DNA fragments ranging from 50 to 500 bp were subcloned into M13 and the sequences were determined by dideoxy sequencing (Sanger et al., 1977) using the Sequenase DNA sequencing kit.

Cell Culture

L2 cells, a rat pulmonary epithelial-like cell line (ATCC CCL 149), were grown as monolayer in Ham's modified F12K medium with 10% fetal bovine serum, and antibiotic antimycotic solution (10 μg/ml glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B) at 37°C in room air with 5% CO_2. When the cells were 95% confluent, they were treated with 0.5 μg/ml E.coli LPS, 2 ng/ml IL-1α, or 10 ng/ml TNF.

RNA Isolation

Total RNA was isolated from animal tissues and cell culture by the acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (1987). Different rat tissues were dispersed in GTC solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol) with a homogenizer, following extraction with 0.1 volume of 2 M sodium acetate, pH 4.0, equal volume of water saturated phenol, and 0.2 volume of chloroform:isoamyl alcohol (49:1).

After centrifugation at 10,000 rpm for 20 min at 4°C, the aqueous phase was transferred to a new tube, mixed with equal volume of isopropanol, and incubated at -20°C for 1 hour. After centrifugation, the RNA pellet was dissolved in GTC solution, transferred to a diethyl pyrocarbonate (DEPC)-pretreated Eppendorf tube, and mixed with equal volume of isopropanol. After isopropanol precipitation, the RNA pellet was dissolved in DEPC-treated water, following by precipitating with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.2 volumes of 100 % ethanol. The RNA pellet was then dissolved in DEPC-treated water and the RNA concentration estimated by absorbance at 260 nm.

For RNA isolation from cell culture, the medium was removed first and the cells were lysed with GTC solution, following the organic extraction as described above.

Northern Analysis

Twenty μg of each RNA sample were fractionated by electrophoresis on 1% agarose-formaldehyde gel as described (Maniatis et al., 1982). After electrophoresis, the RNA gel was treated with 50 mM NaOH, 10 mM NaCl for 45 minutes, 0.1 M Tris (pH 7.5) for 45 minutes, and 50 mM TBE for 30 minutes. The RNA was electrotransferred to a noncharged nylon membrane, and covalently linked to the membrane by UV irradiation (Church & Gilbert, 1984). The membrane was prehybridized for 15 minutes in a buffer containing 1% bovine serum albumin, 1 mM EDTA, 0.5 M sodium phosphate (pH

7.2), and 7% SDS at 65°C. After the radioactive probe was added, the membrane was hybridized overnight and then washed three times in 1 mM EDTA, 40 mM sodium phosphate (pH 7.2), and 0.1% SDS at 65°C. The membrane was then subjected to autoradiography using an intensifying screen at -85°C.

Preparation of Nuclei

Untreated or treated L2 cells were washed with phosphate buffered saline (PBS, 10 mM sodium phosphate, pH 7.4, 150 mM NaCl), and then removed from cell culture plates by trypsinization. The cells were collected in cold nuclear isolation buffer (NIB) containing 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgCl_2$, 0.25 M sucrose, 1 mM DTT, and 60 mM Tris (pH 8.0), and washed twice with the same buffer to remove remaining trypsin. The cells were resuspended in NIB containing 1% Triton X-100 for lysis and the nuclei were collected by centrifugation. After two washes with NIB, the nuclei were resuspended in NIB for DNase I hypersensitivity studies or resuspended in nuclear run-on buffer containing 20% glycerol, 30 mM Tris (pH 8.0), 5 mM $MgCl_2$, 0.15 M KCl, 0.05 mM EDTA, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP and 2.5 mM DTT for nuclear run-on studies.

Preparation of Permeabilized Cells

L2 cells were washed with PBS, trypsinized, and collected in NIB as mentioned in the protocol for the preparation of nuclei. Cells were washed twice with NIB to remove remaining trypsin, then resuspended in 4 ml of cold

NIB containing 0.05% lysolecithin for 1 minute for permeabilization, and finally diluted into 40 ml of cold NIB. After centrifugation, the permeabilized cells were resuspended in NIB for DNase I hypersensitivity studies or in nuclear run-on buffer for nuclear run-on studies.

Nuclear Run-on Studies

The protocol for the nuclear run-on assay was modified from Groudine et al. (1981). After L2 cells were treated with 0.5 μg/ml LPS for 1 hour, the nuclei or permeabilized cells were prepared as described and 25 μl of [α-^{32}P]UTP (3000 Ci/mmol) were added to 400 μl of nuclei suspension (1-2 x 10^7 nuclei). The reaction mixture was incubated at 30°C for 15 minutes, 3 ml of GTC solution were added, and total RNA including radioactive nascent RNA was then isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method described in the protocol of RNA isolation. The radioactive RNA was hybridized with dot blots which contained 3 μg of mouse β-actin, rat Cu/ZnSOD, rat MnSOD cDNA fragments, or linearized pUC 19 plasmid for each dot. After hybridization at 60°C for 1 day in a hybridization solution containing 0.5 M sodium phosphate, 7% SDS, 1 mM EDTA, and 1% BSA, the membrane was washed in 0.004 M sodium phosphate and 0.1% SDS at 65°C and then subjected to autoradiography. After exposure to X-ray film, the induction levels of MnSOD were quantitated by densitometry

using the Bio Image Visage 60 video densitometry system (Millipore/Bio Image).

Genomic DNA Isolation

The genomic DNA from L2 cells, a normal rat pulmonary epithelial-like cell line, was isolated as follows. The medium was removed and cells were lysed in a buffer containing 100 mM EDTA, 1% SDS, and 50 μg/ml proteinase K. After incubation at 56°C for 3 hr, the genomic DNA was extracted with phenol/chloroform and precipitated with ethanol. The genomic DNA sample was treated with 100 μg/ml RNase to remove RNA, followed by ethanol precipitation. The purified genomic DNA was resuspended in TE solution (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0) and the concentration determined by the absorbance at 260 nm.

DNase I Treatment & Genomic DNA Isolation

Aliquots (0.3 ml) of approximately 1 x 10^7 nuclei or permeabilized cells were digested with increasing concentrations of DNase I at 37°C for 4 minutes. The reactions were terminated by adding to final concentration of 1% SDS, 100 mM EDTA (pH 8.0), and 50 μg/ml proteinase K. The genomic DNA was isolated by incubation at 56°C for 3 hours followed by organic extraction and DNA precipitation. The genomic DNA sample was treated with 100 μg/ml RNase to remove remained RNA, followed by ethanol precipitation. The purified genomic DNA was resuspended in TE solution and the concentration determined by the absorbance at 260 nm.

Southern Analysis

Three gel systems have been applied for the Southern analysis to achieve the resolution desired: 1% agarose gel in TAE buffer to resolve the DNA fragments ranging from 0.5 to 15 kb, 2.5% Metaphor agarose gel in TAE buffer to resolve the DNA fragments ranging from 0.1 to 1 kb, and 4% polyacrylamide gel in TBE buffer to achieve the similar resolution as 2.5% Metaphor agarose.

For the 1% agarose gel, 10 μg of purified genomic DNA were digested with appropriate restriction endonucleases for 3 hours, followed by ethanol precipitation, and fractionated on a 1% agarose gel in TAE buffer for overnight. The DNA was denatured and then electrotransferred to a noncharged nylon membrane for 2 hours. The DNA was crosslinked to the membrane with UV light.

For the 2.5% Metaphor agarose gel, 15 μg of purified genomic DNA were digested with appropriate restriction endonucleases, and fractionated on a 2.5% Metaphor agarose gel in TAE buffer for overnight. The DNA was denatured, and neutralized as for 1% agarose gel. After electrotransferred to a noncharged nylon membrane for 2 hours, the DNA was UV crosslinked to the membrane.

For the 4% polyacrylamide gel, 20 μg of purified genomic DNA were digested with appropriate restriction endonucleases, and fractionated on 4 % native polyacrylamide gel in TBE buffer (acrylamide: bisacrylamide = 40:1). The

polyacrylamide gel was denatured by boiling for 10 minutes in a sealed plastic bag (Pauli et al., 1988). The DNA was electrotransferred for 45 minutes to a noncharged nylon membrane and covalently crosslinked to the membrane with UV light.

An identical hybridization method has been applied to all the membranes generated. The membrane was prehybridized for 15 minutes in a buffer containing 1% BSA, 1 mM EDTA, 0.5 M sodium phosphate (pH 7.2), and 7% SDS at 65°C. After the radioactive probe was added, the membrane was hybridized overnight and then washed three times in 1 mM EDTA, 40 mM sodium phosphate (pH 7.2), and 1% SDS at 65°C. The membrane was then subjected to autoradiography with an intensifying screen at -85°C.

Preparation of Probe

For Northern and Southern analysis, [^{32}P]-dATP labeled probes were generated using a random primer DNA labeling system. Briefly, DNA fragments were denatured by boiling for 5 minutes, and then cooled on ice for 5 minutes. Random primers, dCTP, dGTP, dTTP, [^{32}P]-dATP and DNA polymerase were added, and the reaction mixture was incubated at room temperature for 3 hours. The labeled probe was purified through a G-50 column. The DNA fragments used for probe preparation were isolated by restriction digestion of various subclones of the rat Cu/ZnSOD cDNA, rat MnSOD cDNA, or rat MnSOD gene.

CHAPTER 3
RAT COPPER/ZINC SUPEROXIDE DISMUTASE GENE:
ISOLATION, CHARACTERIZATION, AND SPECIES COMPARISON

Introduction

Cells are protected from free radical damage by a chemical and enzymatic antioxidant defense system. The superoxide dismutases (SOD, E.C.1.15.1.1.), which dismutate the superoxide anion into oxygen and hydrogen peroxide, are thought to play a vital role in protecting cells from free radical damage (For reviews see Bannister et al., 1987; Fridovich, 1989). According to the metals found in their active centers, SODs from a wide range of organisms fall into three types: copper-zinc (Cu/ZnSOD), manganese (MnSOD), and iron (FeSOD).

Cu/ZnSOD is found mainly in the cytosol of eukaryotes and in chloroplasts. Rat Cu/ZnSOD protein is a dimer consisting of two identical subunits. Each subunit has a single Cu and Zn atom with a molecular mass of 17 kDa (Steffens et al., 1986). Cu/ZnSOD activity and mRNA level has been widely studied in various species including rat, mouse and human. Our own studies on the expression of the SODs indicate that Cu/ZnSOD mRNA levels, unlike MnSOD, remained unchanged in response to stimulants such as

lipopolysaccharide, interleukin-1, tumor necrosis factor (Visner et al., 1989), and interleukin-6 (Dougall & Nick, 1991). We have, however, observed striking changes in Cu/ZnSOD mRNA and protein levels during the differentiation of 3T3-L1 fibroblasts to adipocytes, which patterns the increases in triglycerides associated with adipogenesis (unpublished results). Along similar lines, it has been documented that the level of Cu/ZnSOD is also regulated during animal development, cell differentiation, and cell cycling. In rat lung, the concentration of Cu/ZnSOD mRNA peaked in late gestation, with a subsequent postnatal decrease (Hass & Massaro, 1987). During differentiation of human leukemia cell lines into macrophages and/or granulocytes, the steady-state mRNA level and the rate of transcription of Cu/ZnSOD decreased (Saito, 1989). In addition, it was shown that Cu/ZnSOD mRNA was very low during the G_0/G_1 phase, peaked during the S phase, and decreased as the cells progressed through their cycles (Kong et al., 1993).

Recently, researchers have linked mutations in the Cu/ZnSOD gene to a herediatary form of a motor neuron disease, amyotrophic lateral sclerosis (ALS) (Rosen et al., 1993; Deng et al., 1993). ALS, also known as Lou Gehrig's disease, is a progressive paralytic disorder caused by degeneration of large motor neuron nuclei of the brain and spinal cord. The genetics studies have demonstrated that

mutations in Cu/ZnSOD gene disrupt the three-dimensional structure of this enzyme, causing the reduction in both its stability and activity. It has been hypothesized that ALS and other neurodegenerative disorders may be caused by damage done to the nerve cells by free radicals. The isolation and characterization of Cu/ZnSOD gene is therefore important for understanding the mechanism of gene expression as well as shedding some light on the diseases associated with it.

In this chapter, I report the isolation and characterization of rat Cu/ZnSOD cDNA and genomic clones. By comparing the 5' flanking region of the Cu/ZnSOD gene from three different species, rat, mouse (Benedetto et al., 1991), and human (Levanon et al., 1985), a striking identity over 230 bp in the promoter region has been identified which includes several potentially important regulatory elements.

Results and Discussion

Cloning and Sequencing of Rat Cu/ZnSOD cDNA Clones

After immunoscreening of a rat liver cDNA library with anti-rat Cu/ZnSOD antibody and confirmation by hybridization with a human Cu/ZnSOD cDNA probe, a positive clone, pJ500, was purified and used for further cDNA library screening. By using the pJ500 cDNA probe, three additional Cu/ZnSOD cDNA clones were isolated and characterized. The longest clone, C14-2, contains 75 bp of 5' noncoding sequence, 462

bp of coding sequence corresponding to 154 amino acids, and 91 bp of 3' non-coding sequence, for a total of 628 bp. The nucleotide and deduced amino acid sequences of rat Cu/ZnSOD cDNA are shown in figure 3-1.

Tissue Distribution of Rat Cu/ZnSOD

Using the rat Cu/ZnSOD cDNA as a probe, we evaluated the expression of Cu/ZnSOD by Northern blot analysis in six different rat tissues: heart, brain, lung, liver, kidney, and intestine. As shown in Figure 3-2, the Cu/ZnSOD is expressed in all the tissues at varying levels with kidney and liver having the highest Cu/ZnSOD mRNA level. This distribution is consistent with the relative protein concentration and activity of Cu/ZnSOD in these tissues (Asayama & Burr, 1985), demonstrating that the level of this important antioxidant enzyme varies in a tissue-specific manner and may reflect, for instance, in the liver, a role in the detoxification of environmental xenobiotics.

Cloning and Sequencing of Rat Cu/ZnSOD Gene

To ultimately investigate the regulation of Cu/ZnSOD gene expression, a genomic clone, G1-1, containing a 13Kb insert was isolated from a rat genomic library using my rat Cu/ZnSOD cDNA probe, c14-2. A restriction map and the exon positions for this genomic clone are shown in Figure 3-3. This gene spans approximately 6 kb and contains five exons and four introns.

```
                                        30                                  60
CTG CGG CGG CTT CTG TCG TCT CCT TGC TTT TTG CTC TCC CAG GTT CCG AGG CCG CCG CGC

                                        90                                 120
GTC TCC CGG GGA AGC ATG GCG ATG AAG GCC GTG TGC GTG CTG AAG GGC GAC GGT CCG GTG
                    Met Ala Met Lys Ala Val Cys Val Leu Lys Gly Asp Gly Pro Val

                                       150                                 180
CAG GGC GTC ATT CAC TTC GAG CAG AAG GCA AGC GGT GAA CCA GTT GTG GTG TCA GGA CAG
Gln Gly Val Ile His Phe Glu Gln Lys Ala Ser Gly Glu Pro Val Val Val Ser Gly Gln

                                       210                                 240
ATT ACA GGA TTA ACT GAA GGC GAG CAT GGG TTC CAT GTC CAT CAA TAT GGG GAC AAT ACA
Ile Thr Gly Leu Thr Glu Gly Glu His Gly Phe His Val His Gln Tyr Gly Asp Asn Thr

                                       270                                 300
CAA GGC TGT ACC ACT GCA GGA CCT CAT TTT AAT CCT CAC TCT AAG AAA CAT GGC GGT CCA
Gln Gly Cys Thr Thr Ala Gly Pro His Phe Asn Pro His Ser Lys Lys His Gly Gly Pro

                                       330                                 360
GCG GAT GAA GAG AGG CAT GTT GGA GAC CTG GGC AAT GTG GCT GCT GGA AAG GAC GGT GTG
Ala Asp Glu Glu Arg His Val Gly Asp Leu Gly Asn Val Ala Ala Gly Lys Asp Gly Val

                                       390                                 420
GCC AAT GTG TCC ATT GAA GAT CGT GTG ATC TCA CTC TCA GGA GAG CAT TCC ATC ATT GGC
Ala Asn Val Ser Ile Glu Asp Arg Val Ile Ser Leu Ser Gly Glu His Ser Ile Ile Gly

                                       450                                 480
CGT ACT ATG GTG GTC CAC GAG AAA CAA GAT GAC TTG GGC AAA GGT GGA AAT GAA GAA AGT
Arg Thr Met Val Val His Glu Lys Gln Asp Asp Leu Gly Lys Gly Gly Asn Glu Glu Ser

                                       510                                 540
ACA AAG ACT GGA AAT GCT GGA AGC CGC TTG GCT TGT GGT GTG ATT GGG ATT GCC CAA TAA
Thr Lys Thr Gly Asn Ala Gly Ser Arg Leu Ala Cys Gly Val Ile Gly Ile Ala Gln

                                       570                                 600
ACA TTC CCT ATG TGG TCT GAG TCT CAG ACT CAT CTG CTG TCC TGC TAA ACT GTA GAA AAA

AAC CAA ACC ATT AAA CTG TAA TCT TAA C
```

Figure 3-1. The nucleotide and deduced amino acid sequences of rat Cu/ZnSOD cDNA.

Total of 628 nucleotide sequence is shown with the numbers on the top. The amino acid codes shown below the nucleotide sequence indicate the deduced amino acid sequence. The polyadenylation signal is underlined.

Figure 3-2. Tissue distribution of rat Cu/ZnSOD mRNA.
Twenty μg of total RNA from different rat tissues were fractionated by electrophoresis, electrotransferred to a nylon membrane, and hybridized to radiolabeled rat Cu/ZnSOD cDNA probe. The tissues shown included: H: heart; B: brain; Lu: lung; Li: liver; K: kidney, and I: intestine.

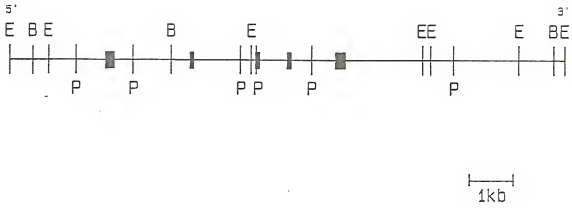


Figure 3-3. Structure and restriction map of the rat Cu/ZnSOD gene.

A graphical representation of the rat Cu/ZnSOD genomic clone illustrating the location of prominent restriction sites and the position of each of the five exons. The exons are represented as black rectangles. The restriction sites are abbreviated as follows: B, BamHI; E, EcoRI; and P, PstI. The approximate scale is designated by the 1 kb marker on the right.

A genomic Southern analysis shown in Figure 3-4A indicates that there is most likely a single gene for this protein in the rat genome. This is most evident in the lane with HindIII digestion, which shows a single band. Figure 3-4B depicts a genomic Southern analysis for comparison to that of my genomic clone, which demonstrates that the restriction map of G1-1 is identical to the Cu/ZnSOD locus in the rat genome.

To further characterize the structure of the rat Cu/ZnSOD gene, the nucleotide sequence of this gene was determined and shown in Figure 3-5. This includes 800bp of 5' flanking sequence, all the exons and part of their flanking introns. The nucleotide sequence of the five genomic exons is identical to that of my rat Cu/ZnSOD cDNA.

The intron-exon junctions were identified by comparing the rat Cu/ZnSOD genomic sequence to the rat Cu/ZnSOD cDNA sequence. Most of the intron-exon junctions conform to the consensus sequences established for intronic donor (5' GTPuAGT3') and acceptor (5'$(Py)_nNPyAG3'$) splice signals (Shapiro & Senapathy, 1987) except the donor sequence at the first intron which contains an unusual 5' GC rather than the highly conserved GT. This unusual 5' splice sequence has been shown to be efficiently utilized in vivo (Fischer et al, 1984).

Several promoter sequences for eukaryotic gene expression which reside upstream from the transcription

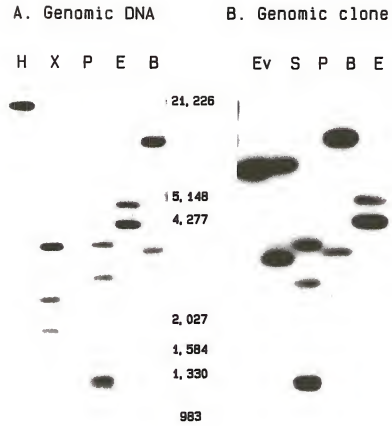


Figure 3-4. Southern analysis.
(A) 10 μg of rat genomic DNA or (B) 2 μg of DNA insert from the rat Cu/ZnSOD genomic clone, G1-1, were digested with various restriction enzymes, fractionated through 1% agarose gel, electrotransferred to a nylon membrane, and hybridized to radiolabeled rat Cu/ZnSOD cDNA probe. The restriction enzymes used: B, BamHI; E, EcoRI; Ev, EcoRV; H, HindIII; P, PstI; S, ScaI; and X, XbaI. The fragment sizes are determined relative to size markers depicted in base pairs.

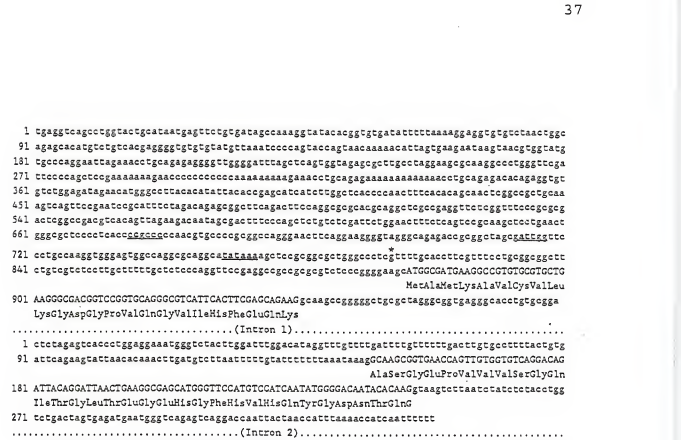
start site (Ho & Crapo, 1987) of rat Cu/ZnSOD gene are underlined in Figure 3-5. At position -23 to -29 upstream from the mRNA start site there is a hexanucleotide TATAAA, the so-called TATA box, which is essential for accurately positioning the start of transcription. At position -64, relative to the mRNA start site, there is an inverted CCAAT sequence.

Comparison of the Gene Structure of Rat, Mouse, and Human Cu/ZnSOD

Based on the amino acid identity between different species, Cu/ZnSOD has been highly conserved during evolution (Bannister et al., 1987). When comparing the rat, mouse (Benedetto et al., 1991), and human (Levanon et al., 1985) Cu/ZnSOD gene several important correlations can be found. First, the exon-intron organization of rat, mouse, and human Cu/ZnSOD gene is identical. All three genes encode a protein of 154 amino acids and have four introns dividing their protein-coding sequence into five exons. The interrupted positions of all the intron pairs along the coding sequence are conserved. For instance, in all three species, exon 1 contains the 5' untranslated region and the nucleotides which encode the first 24 amino acids and all remaining exons contain the same amino acid intervals. This level of conservation in intron/exon structure over the evolutionary span between rodents and man indicates that they are derived from a common ancestral gene which has

Figure 3-5. Nucleotide and amino acid sequence of rat Cu/ZnSOD gene.

A representation of sequence from the 5' flanking region, all the exons and sequences in the flanking introns of the rat Cu/ZnSOD gene is depicted. The coding sequence within each of the five exons are capitalized and the corresponding amino acid sequence shown and numbered below. The transcription start site is indicated by an asterisk (*) and several promoter elements, such as the TATA box, CCAAT box, and a GC-rich motif, are underlined.


MetAlaMetLysAlaValCysValLeu
LysGlyAspGlyProValGlnGlyValIleHisPheGluGlnLys
(Intron 1)
AlaSerGlyGluProValValValSerGlyGln
IleThrGlyLeuThrGluGlyGluHisGlyPheHisValHisGlnTyrGlyAspAsnThrGlnG
(Intron 2)
lyCysThrThrAlaGlyProHisPheAsnProHisSerLysLysHisGlyGl
yProAlaAspGluGluAr
(Intron 3)
gHisValGlyAspLeuGlyAsnValAlaAlaGlyLysAspGlyValAlaAsnValSerIle
GluAspArgValIleSerLauSerGlyGluHisSerIleIleGlyArgThrMetVal
(Intron 4)
ValHisGluLysGlnAspAspLeuGlyLysGlyGlyAsnGluGluSerThrLysThrGlyAsnAlaGlySerA
rgLeuAlaCysGlyValIleGlyIleAlaGlnStop

retained its genomic structure due to strong selective pressure.

The sequences of intron-exon junctions from rat, mouse, and human Cu/ZnSOD genes were compared and shown in Figure 3-6A. All of the intron-exon sequences followed the consensus sequences for RNA splicing except the unusual 5' GC donor sequence of the first intron. This unusual GC donor sequence exists in the first intron of all three species. The retention of this splice site anomaly may result from the fact that structural or functional domains of proteins are often encoded by discrete exons and, therefore, due to the importance of the intron-exon junction relative to the protein structure, this GC donor sequence has been, somehow, conserved. However, since this GC dinucleotide is part of an intron, not exon 1, it seems unlikely that it would be conserved for structural reasons. We, therefore, postulate that in addition to its function as a splicing signal, the GC dinucleotide may also have a key role as part of a potential regulatory factor binding site.

Benedetto et al. (1991) presented the structure of the murine Cu/ZnSOD gene and compared the promoter region of mouse and human Cu/ZnSOD gene using the translation start site to align the sequences. This resulted in the conclusion that this region has a low level of identity between these two species. In addition, all the important

Figure 3-6. Comparison of genomic organization of rat, mouse, and human Cu/ZnSOD genes.

(A) Comparison of the splice junctions for the rat, mouse, and human Cu/ZnSOD genes.
Sequences of the four intron/exon junctions from rat, mouse, and human Cu/ZnSOD genes were compared. All the donor and acceptor sequences were underlined, except the unusual 5' GC donor sequence in the first intron, which was double underlined.

(B) Nucleotide alignment of the rat (R), mouse (M), and human (H) Cu/ZnSOD promoter region. Approximately 230 nucleotides beginning immediately upstream from the rat, mouse, and human Cu/ZnSOD transcription start sites (indicated by an asteisk) were aligned by computer analysis using Beckman Microgenie software. Regions of homology which contain sequences known to regulate transcription are double-underlined and identified. Also, additional regions of identity were highlighted by underlining.

(A)

		Donor (GT) 5' 3'	Acceptor (AG) 5' 3'
Intron 1	Rat	AAGGCAAGC.....	.....TTAAATAAAGGC
	Mouse	AAGGCAAGG.....	.....TTAAATCAAGGC
	Human	AAGGCAAGG.....	.....TTTCTTAAAGGA
Intron 2	Rat	AAGGTAAGT.....	.....TGGTAAATAGGC
	Mouse	AAGGTAGGT.....	.....TGGTAAATAGGC
	Human	CAGGTGGGT.....	.....TTATAAATAGGC
Intron 3	Rat	GAGGTGAGC.....	.....TTAATGTTAGGC
	Mouse	GAGGTGAGG.....	.....TTAATGTTAGGC
	Human	GAGGTAACA.....	.....TTTCATATAGGC
Intron 4	Rat	GTGGTAAGT.....	.....CTTTTCAAAGGC
	Mouse	GTGGTAAGT.....	.....CTTTTCAAAGGT
	Human	GTGGTAAGT.....	.....TTTTTTACAGGT

(B)

```
-225 TCACAGTTAGAAGACAATAGCGAC--TTTGCCGAGCTGAGGCTC--CTGGGGAACTTTCTC   M
     ||||||||||||||||||||||||  ||||||||||| | |||   || ||||||||||
-228 TCACAGTTAGAAGACAATAGCGAC--TTTCCGAGCTCTGTCTCGATTCTGGAACTTTCTC   R
     | | |   ||       | | ||  ||||   | ||||   || |||  |
-235 TGAAAAGAAGGTTGTTTTCTCACACAGTTTCGGGGTTCTG-GACGTTTCCCGGCTGCGGGG   H

-169 AGTC-GCAAGCTCCAGGAGCTCGAGCTATCCTCGGGCCCCGCCCCCAGGCTCCCCCGCGGC   M
     |||| |||||||||| | |    | |||  ||||  ||||||||||| ||||||||||||
-170 AGTCGGCAAGCTCCTGAACTGGCGGCTCCCCTCA-CCCCGCCCCCAACGTGCCCCGCGGC   R
       | | |  |||| |  |   |||  |||||    ||||||||   | | ||| ||  |
-176 CGGGGGGAGTCTCGGGCGC-ACGGG-GCCCCTTGG-CCCGCCCCAGTCATTCCCGGGCCAC   H
                                          GC-rich motif

-109 CAGGGAGC---TCCAC--GAA--GGGCGGGCGGAGGCCGCGGGTAGCGATTGGTTCCGTG   M
     ||||||  |   | ||   |||  ||  |||| ||| ||||||  ||||||||||||||  ||
-111 CAGGGAAC---TTCAG--GAAGGGGTAGGGCAGAGACCGCGGCTAGCGATTGGTTCCCTG   R
       | || |      | |  | |||||  |||| |||  || |    || |||||||   |
-119 TCGCGACCCGAGGCTGCCGCAGGGGGCGGGCTGAGCGGGTGCGAGGCGATTGGTTTGGGG   H
                                                  Inverted CCAAT box

-56  CCAAGGTGGGCGTGGTCAGACT--CAGGCCTATAAAAGCTCCGTGGCG-CCAGGGCCTCG   M
     ||||||||||  |||| ||| |   |||||  ||||||||||||| |||| |  || |||||
-56  CCAAGGTGGGAGTGGCCAGGGG--CAGGCATATAAAAGCTCCGCGGCG-CTGGGCCCTCG   R
     |||  ||||| | |||  || |  | ||| ||||||     ||||| | | |||  || |
-59  CCAGAGTGGGCGAGGCGCGAGGTCTGGCCTATAAAGTAGTCGCGGAGACGGGGTGCTGG   H
                                  TATA box                      *
```

promoter elements for transcriptional initiation, such as the TATA and CCAAT box, could not be aligned.

In Figure 3-6B, I aligned the 5' flanking sequence for rat, mouse and human Cu/ZnSOD genes using the mRNA transcription start site. This comparison has revealed a significant degree of identity in this region: 84% between rat and mouse; 56% between rat and human; and 54% between human and mouse. Most strikingly, major blocks of identity encompass the TATA box, the inverted CCAAT box, as well as several highly conserved repeats in the region upstream of these general promoter elements. The high level of sequence identity in the promoter region of Cu/ZnSOD gene between species strongly suggests that there is a common mechanism for the regulation of the Cu/ZnSOD gene in these species. These conserved repeats most likely serve as binding sites for trans-acting factors, which may play a role in regulating the basal expression level of the Cu/ZnSOD gene in different tissues.

Specifically, there are three GC-rich regions centered at approximately -90, -115 and -130 which are conserved and may function as SP-1 binding sites. This promoter alignment has also identified a region from -160 to -225 with a strong identity between the two rodent species, which contains an imperfect inverted repeat centered at -175. Unfortunately, any analysis further upstream is limited by the lack of sequence information from the mouse and human genes. This

high level of identity in the 5′ flanking region is unusual for such divergent species, without some form of selective pressure. The isolation and characterization of rat Cu/ZnSOD gene is, therefore, important to further understand the expression and regulation of this gene and the diseases associated with it.

CHAPTER 4
CHROMATIN STRUCTURE ANALYSIS OF RAT MANGANESE SUPEROXIDE DISMUTASE GENE

Introduction

Superoxide dismutases (SODs), which catalyze the dismutation of superoxide anion to hydrogen peroxide and oxygen, is one of the biochemical defense mechanisms that organisms have evolved to protect form the damaging effect of oxygen free radicals. Strong evidence has been presented indicating that many of the cell alterations seen in normal aging and in various diseases are due to oxidative damage from active oxygen species. Study of these enzymes is therefore of potential clinical interest. Three forms of SOD have been found in eucaryotes: cytoplasmic copper/zinc SOD (Cu/ZnSOD), extracellular copper/zinc SOD (EC-Cu/ZnSOD), and mitochondrial manganese SOD (MnSOD). It has been demonstrated that while Cu/ZnSOD in most cases is constitutively expressed, the MnSOD is highly regulated.

Most mammalian MnSODs are tetrameric enzymes with molecular weights of approximately 80 KD. This enzyme contains manganese at its active site and is mainly located in the matrix of mitochondria. The cDNA and genomic clones of rat MnSOD have been isolated and characterized (Ho et

al., 1991; Hurt et al., 1992). The rat MnSOD cDNA sequence encodes a protein of 222 amino acids which includes a putative mitochondrial targeting sequence of 24 amino acids. We have also demonstrated that the multiple mRNA species detected in Northern analysis are generated by alternate polyadenylation from a single functional gene (Hurt et al., 1992).

Previously, we have shown that the steady-state mRNA levels of MnSOD, but not Cu/ZnSOD, could be dramatically induced in L2 cells, a rat pulmonary epithelial-like cell line, in response to three inflammatory mediators: LPS, IL-1, and TNF (Visner et al., 1989). This increase in MnSOD mRNA level was detected after 1 hour treatment, and was maximal after 8 hours treatment. This induction was blocked by actinomycin D, a transcription inhibitor, indicating that increase in MnSOD mRNA by LPS, IL-1, and TNF might result from an increase in transcription of the MnSOD gene. To further verify whether stimulus-dependent increases in MnSOD mRNA levels reflect an increased transcriptional activity of the gene or changes in the inherent stability of the mRNA, I have assayed relative amounts of nascent transcripts by nuclear run-on studies.

I have also investigated the regulatory mechanism by evaluating changes in MnSOD chromatin structure using DNase I hypersensitivity studies. The eukaryotic chromatin structure is the result of packaging the genetic material

into several levels of higher order structures. First, the DNA is wrapped approximately 1.8 turns around the nucleosome core. An protein octamer composed of histones H2A, H2B, H3, and H4 makes up the nucleosome core particle, with histone H1 binding across the entry and exit points of the DNA from the nucleosome core (Richmond et al., 1984). This 10 nm "beads on a string" fiber is then wound up into a 30 nm solenoid filament with six nucleosomes per turn (Felsenfeld & McGhee, 1986). The 30 nm fiber is further compacted into consecutive loops of chromatin (Rattner & Lin, 1985) which appear to attach to either chromosomal protein scaffolds or nuclear matrix material.

Eukaryotic chromosomes participate in transcription and replication which occur through specific interactions between nuclear proteins and chromosomal DNA sequences. Recognition of specific DNA sequences by proteins requires accessibility. It has been shown that active (or potentially active) genes undergo some dramatic changes in their chromatin structure relative to quiescent regions of the genome. One important indication of altered chromatin structure within an active gene region is heightened sensitivity to DNase I (reviews in Gross, 1988; Elgin, 1988). When bulk chromatin is subjected to digestion using low concentration of nuclease, such as DNase I, a ladder of fragments differing by about 200 bp in length is generated. This pattern reflects the placement of successive

nucleosomes along the chromatin fiber. However, some regions of the actively transcribed genes are not uniformly degraded by DNase I but contain local sites two orders of magnitude more sensitive towards DNase I cleavage than inactive chromatin and hence are called DNase I hypersensitive sites. This greater sensitivity toward nuclease strongly suggests an unfolding or disorganization of the chromatin upon gene activation.

In chromatin, nucleosome-free regions known as nuclease hypersensitive sites are believed to represent the "open windows" that allow enhanced access of crucial resident cis-acting DNA sequences to trans-acting factors. These accessible regions are operationally defined by their pronounced sensitivity to nuclease cleavage or chemical modification. For general applications DNase I is the enzyme of choice because of its high selectivity for nucleosome-free regions and relatively low DNA sequence specificity (Galas & Schmitz, 1978). In addition, dimethyl sulfate (DMS) (Ephrussi et al., 1985) or UV-light (Becker et al., 1989) treatment of living cells have been very effective in probing hypersensitive site substructure at nucleotide-level resolution.

Several types of DNase I hypersensitive sites have been described: constitutive, inducible, and tissue-specific. Constitutive hypersensitive sites are usually found in the promoter regions of housekeeping genes that are expressed at

basal rates. The inducible hypersensitive sites are often associated with differentiation (Crowder & Merlie, 1988), development (Kikuch et al., 1992), and stimulants such as steroid (Hecht et al., 1988) or growth hormone (Bichell et al., 1992). Tissue-specific DNase I HS sites have been observed in the lung-specific surfactant protein B gene (Bohinski et al., 1993) and the tyrosine aminotransferase gene (Becker et al., 1984).

DNase I hypersensitive sites frequently map near the 5' end of actively transcribed genes. However, hypersensitive sites in the gene itself or at the 3' end of the gene have been found. Various studies have shown that DNase I hypersensitive sites in chromatin are generally nucleosome-free (McGhee et al., 1981; Weiss et al., 1986). Analysis at higher resolution indicated, however, that while such sites always include segments of protein-free DNA, they can also contain internal regions associated with nonhistone chromosomal proteins such as RNA polymerase II, and transcription factors (Johnson & McKnight, 1989; Mitchell & Tjian, 1989).

Results and Discussions

Transcriptional Regulation of Rat MnSOD Gene

It has been shown that the steady-state mRNA levels of rat MnSOD are regulated by three inflammatory mediators: LPS, IL-1, and TNF (Visner et al., 1989). A 24 hour time

course of LPS treatment on L2 cells, a normal rat pulmonary epithelial-like cell line, showed that control L2 cells exhibited a very low constitutive level of expression of MnSOD, while the L2 cells exposed to LPS manifested a dramatic induction of MnSOD. The initial increase of MnSOD mRNA level was observed after 1 hour of LPS exposure with a maximal induction of 18-to 23- fold after 8 hours. Treatments of IL-1 and TNF caused similar levels of induction relative to LPS in L2 cells.

The induction of MnSOD mRNA by LPS, IL-1, and TNF could be a consequence of a direct increase in transcriptional rate, the prolongation of MnSOD mRNA half-life, or both. To address the mechanism for this increase, L2 cells were treated with actinomycin D, a RNA synthesis inhibitor, in combination with LPS. When L2 cells were treated with actinomycin D alone, little or no change of the control levels of MnSOD mRNA was observed. However, when the cells were co-treated with LPS and actinomycin D, actinomycin D totally inhibited the MnSOD mRNA induction by LPS. Similar results have been obtained when cells were co-treated with actinomycin D in combination with IL-1 or TNF. These data strongly suggest that induction of MnSOD mRNA level by LPS, IL-1, and TNF is at least partially transcriptionally dependent.

To further illustrate that the regulation of rat MnSOD gene by LPS, IL-1, and TNF is transcriptional, nuclear run-

on studies have been performed to assay for the relative amounts of nascent transcripts of MnSOD. Using the nuclear run-on technique, newly synthesized RNA can be labeled to high specific activity in isolated nuclei, while this is difficult to accomplish in intact cells. The nuclear run-on studies allow the direct measurement and comparison of specific gene transcription in cells treated with different stimulants, therefore, they are often used to assess whether changes in the mRNA levels of a particular gene reflect a change in the transcriptional activity of this gene or a change in mRNA stability.

For nuclear run-on studies, 95% confluent L2 cells were treated with 0.5 μg/ml LPS for 1 hour and the nuclei from control and treated cells were prepared by lysing the cells with 1% Triton X-100. Radioactive UTP was added to the nuclei preparation and the transcription was allowed to continue at 30°C for 15 minutes. Total RNA, including radioactive nascent RNA, was isolated and used to hybridize a dot blot containing MnSOD, Cu/ZnSOD, actin and pUC19 DNA. The relative intensities of the radioactive RNA which hybridizes to the specific MnSOD cDNA fragments will represent the relative amount of nascent transcripts of MnSOD in different treatments. Also, two internal controls, rat Cu/ZnSOD cDNA and mouse actin cDNA, are included to correct any variability in different preparations. In addition, pUC19 plasmid, which all the cDNA fragments were

cloned into, is used as negative control. After exposure to X-ray film, the induction level of MnSOD transcriptional rate was calculated by densitometry. As shown in figure 4-1, the induction level of MnSOD transcriptional rate in LPS-treated cells is 3.1 fold higher than that in control cells, using actin as an internal control. The increased synthesis of nascent MnSOD mRNA transcripts after LPS treatment indicates that this inflammatory mediator transcriptionally regulates the rat MnSOD gene.

However, when comparing these results to the maximal induction of 18- to 23- fold in MnSOD mRNA level after LPS treatment, a 3.1 fold increase in transcriptional rate can not account for the total increase in mRNA level. Several possible explainations can be considered. First, the artificial transcriptional system inherent in nuclei preparation may cause the loss of transcriptional factor(s), therefore, the transcriptionl rate measured here only partially represented the real transcriptional rate change. Second, two regulatory mechanisms, transcriptional rate increase and mRNA stabilization, were both involved in the induction of rat MnSOD gene by these inflammatory mediators. Since the maximal transcriptional rate increase of 3.1 fold was observed after 1 hour LPS treatment, the maximal increase of 18- to 23- fold in the mRNA level observed after 8 hours LPS treatment might be a combination of transcriptional rate increase and mRNA stabilization.

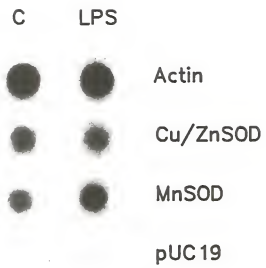


Figure 4-1. Transcriptional regulation of rat MnSOD gene by LPS.

After L2 cells were treated with 0.5 μg/ml LPS for 1 hour, nuclei from control and treated cells were isolated and nuclear run-on studies were performed at 30°C for 15 minutes. Total RNA, including the radioactive nascent RNA, were collected and hybridized with the dot blots containing the cDNA fragments of actin, Cu/ZnSOD, and MnSOD, and linearized pUC19 plasmid. After wash, the membranes were exposed to x-ray film with intensifying screen at -85°C for 1 week.

Third, the correlation between the increase in the nascent RNA synthesis rate measured by nuclear run-on and the increase in mRNA steady-state level are not well documented. Therefore, nuclear run-on studies can only be used as qualitative, but not quantitative, measurement for nascent RNA synthesis rate change.

Chromatin Structure of Rat MnSOD Gene Determined by DNase I hypersensitivity Studies

Since the nuclear run-on studies have indicated that rat MnSOD gene is at least partially regulated at the transcriptional level by LPS, further chromatin structure analysis has been performed using DNase I hypersensitivity studies. An important characteristic of active genes is their preferential sensitivity towards digestion with DNase I at a few discrete sites which have been referred to as DNase I hypersensitive (HS) sites. DNase I HS sites have been found in the promoter regions of actively expressed genes, potentially active genes, and genes that were once active. The DNase I sensitivity is not restricted to the coding region of active genes but extends into their flanking chromatin and gradually drops towards the very 5'- and 3'-end of each locus. DNase I hypersensitivity has been well characterized for the Drosophila heat shock genes (Wu et al., 1979; Wu, 1980), the chicken lysozyme gene (Fritton et al., 1984; Bonifer et al., 1991), and the globin gene cluster (Tuan et al., 1985; Chung & Whiteley, 1993; Philipsen et al., 1993).

Rat MnSOD genomic clones have been isolated and characterized by our group and others (Ho et al., 1991; Hurt et al., 1992). As illustrated in figure 4-2, this gene contains 5 exons which spans approximately 7 Kb of genomic DNA. The restriction map and sequences of the rat MnSOD gene available allowed me to further explore the chromatin structure associated with the regulation of this gene by DNase I hypersensitivity studies.

The method I used to map hypersensitive sites is DNase I digestion of nuclei followed by indirect end-labeling. After the appropriate restriction endonuclease(s) was chosen to generate a suitable size restriction fragment, the probe abutting the restriction site was used to recognize a set of daughter fragments defined at one end by the restriction site and at the other end by the DNase I hypersensitive site(s). These DNase I cleavage points were mapped by determining subfragment sizes on the x-ray film after hybridization.

Several criteria for designing probes have been considered. First, the DNA fragment chosen as a probe should be about 300 to 500 bp long and abut the restriction site that generates a suitable size restriction fragment. Second, all the probes used for DNase I hypersensitivity studies must be screened to avoid any repetitive elements. DNA fragments located in different positions of the rat MnSOD gene have been tested for their appropriateness as

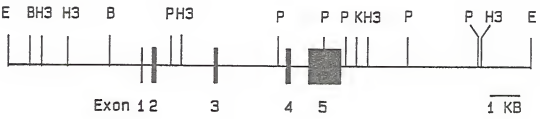


Figure 4-2. Genomic structure of rat MnSOD gene.
A graphical representation of the rat MnSOD genomic clone illustrating the location of the restriction sites and the position of each of the five exons. The exons are represented as black rectangles. The restriction sites are abbreviated as follows: B: BamHI, E: EcoRI, H3: HindIII, K: KpnI, P: PstI. The approximate scale is designated by the 1 kb marker on the right.

probes to study the chromatin structure. As shown in figure 4-3, a total of fourteen DNA fragments have been tested, only five of them are free of repetitive elements. Figure 4-4 is an illustration of the hybridization pattern differences observed with DNA fragment containing repetitive elements and DNA fragment free of repetitive elements. If the DNA fragment tested is free of repetitive elements, specific band(s) corresponding the rat MnSOD genomic structure will be recognized by the probe, as shown in figure 4-4A. If the DNA fragment tested contains repetitive elements, nonspecific bands not corresponding to the rat MnSOD gene will show up, as shown in figure 4-4B.

Four DNA fragments which are free of repetitive elements have been subcloned and used in the DNase I hypersensitivity studies. As shown in Figure 4-3, probe PK is a 0.43 Kb PstI-KpnI DNA fragment which abuts the PstI and KpnI restriction sites at the 3' end of the rat MnSOD gene. Probe KH is a 0.45 Kb KpnI-HindIII DNA fragment which abuts the KpnI and HindIII restriction sites at the 3' end of this gene, which is further 3' than probe PK. Probe BH is a 0.38 Kb BamHI-HincII DNA fragment that abuts the BamHI and HincII restriction sites where the BamHI site exists about 1.1 Kb 5' from the transcription initiation site. Probe HN is a 0.32 Kb HincII-NcoI DNA fragment that abuts the HincII and NcoI restriction sites where the HincII site exists about 0.7 Kb 5' from the transcription initiation site.

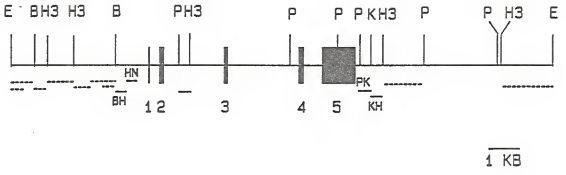


Figure 4-3. Probes design for DNase I hypersensitivity studies.

A graphical representation of the rat MnSOD genomic clone illustrating the location of the DNA fragments tested for repetitive elements. Dotted lines below the restriction map indicate the DNA fragments containing repetitive elements. Full lines below the restriction map indicate DNA fragments free of repetitive elements. Four of the DNA fragments have been subcloned and used in DNase I hypersensitivity studies, namely, probe PK (PstI to KpnI), probe KH (KpnI to HindIII), probe BH (BamHI to HincII), and probe HN (HincII to NcoI).

Figure 4-4. Repetitive elements screening.
Purified genomic DNA from L2 cells was digested with restriction endonucleases, E: EcorI, H: HindIII, and P: PstI. The DNA was fractionated on 1% agarose gel and transferred to noncharged nylon membranes. The membranes were hybridized with (A) probe generated from a DNA fragment free of repetitive elements and (B) probe generated from a DNA fragment containing repetitive elements. After washing, the membranes were exposed to x-ray film with intensifying screen at -85°C for overnight.

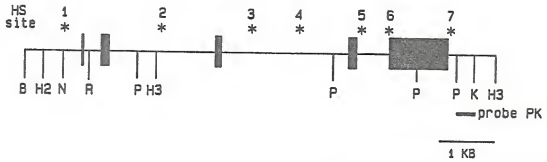
To examine the chromatin structure of the rat MnSOD gene, nuclei were isolated from L2 cells treated with LPS, IL-1, or TNF, and incubated with increasing concentrations of DNase I at 37°C for 4 minutes. The genomic DNA was purified by organic extraction, digested with the appropriate restriction endonuclease, fractionated on a 1% agarose gel, and analyzed by Southern blot hybridization. In figure 4-5, purified genomic DNA was digested with the restriction endonuclease, KpnI, fractionated on a 1% agarose gel, electrotransferred to a nylon membrane, and hybridized with probe PK. When no DNase I was added, probe PK recognized a 14.5 Kb KpnI fragment in the genome, as shown in figure 4-5, lane 0. In control cells, with increasing concentrations of DNase I, a total of seven HS sites were revealed by probe PK. The size of each DNase I HS subfragment has been determined according to the size marker. HS site 1 exists in the promoter region of the rat MnSOD gene and the HS sites 2-7 exist within the gene itself. These seven HS sites have been positioned and shown with the restriction map of rat MnSOD gene in figure 4-5. In LPS, IL-1, and TNF treated cells, with increasing concentrations of DNase I, the same seven HS sites have been observed.

To further detail the HS sites located at the 3' end of rat MnSOD gene, probe KH was designed. In figure 4-6, purified genomic DNA was digested with the restriction

Figure 4-5. DNase I hypersensitive sites in rat MnSOD gene (KpnI and probe PK).

After L2 cells were treated with 0.5 μg/ml LPS, 2 ng/ml IL-1 or 10 ng/ml TNF for 4 hours, the nuclei were prepared and incubated with increasing concentrations of DNase I (0: 0 U/ml, 1: 100 U/ml, 2: 150 U/ml, 3: 200 U/ml and 4: 300 U/ml) at 37° C for 4 minutes and genomic DNA was isolated from each treatment. Ten μg of genomic DNA from each sample was restricted with KpnI and subjected to 1% agarose electrophoresis. The probe PK was used to reveal the DNase I HS sites. The seven HS sites are numbered on the right with a * designating their positions.

The restriction map of rat MnSOD gene shown above illustrates the relative positions of the probe used and the HS sites revealed.


HS site
1
2
3
4
5
6
7
B H2 N R P H3 P P P K H3
probe PK
1 KB

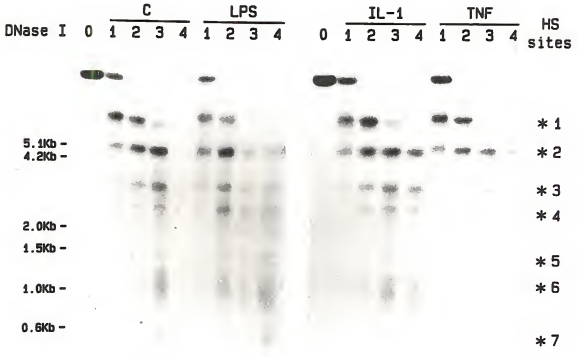
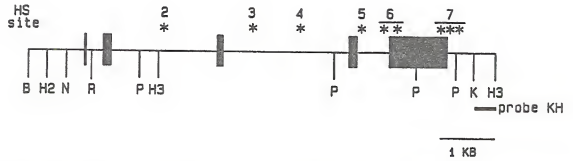
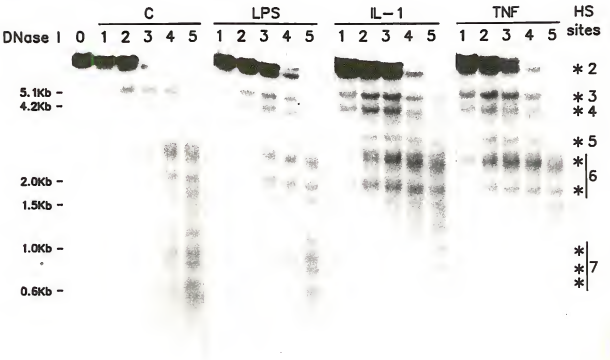

DNase I
C
LPS
IL-1
TNF
0 1 2 3 4 1 2 3 4 0 1 2 3 4 1 2 3 4
HS sites
5.1Kb
4.2Kb
2.0Kb
1.5Kb
1.0Kb
0.6Kb
*1
*2
*3
*4
*5
*6
*7

Figure 4-6. DNase I hypersensitive sites in rat MnSOD gene (HindIII and probe KH).

Genomic DNA was obtained as described in figure 4-5 and the DNase I concentrations used were 0: 0 U/ml, 1: 50 U/ml, 2: 75 U/ml, 3: 100 U/ml, 4: 150 U/ml, and 5: 200 U/ml. Ten μg of genomic DNA was digested with HindIII, fractionated on 1% agarose gel, electrotransferred, and hybridized with probe KH to reveal the DNase I HS sites.

The restriction map of rat MnSOD gene shown above illustrates the relative positions of the probe used and the HS sites revealed.


HS site
2
3
4
5
6
7
B H2 N R P H3 P P P K H3
probe KH
1 KB


C
LPS
IL-1
TNF
HS sites
DNase I 0 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
5.1Kb –
4.2Kb –
2.0Kb –
1.5Kb –
1.0Kb –
0.6Kb –
2
3
4
5
6
7

endonuclease, HindIII, fractionated on a 1% agarose gel, electrotransferred to a nylon membrane, and hybridized with probe KH. With no DNase I treatment, this probe recognized a 6 Kb HindIII fragment from the genome, as shown in figure 4-6, lane 0. When hybridized with DNase I-treated DNA samples, a total of six HS sites were detected in control as well as LPS, IL-1, and TNF treated cells. This probe would not detect HS site 1, but served to expand HS sites 2-7 existing between exon 2 to the end of this gene. Analysis of sites 6 and 7 also revealed that these sites were composed of two or three HS subsites, respectively.

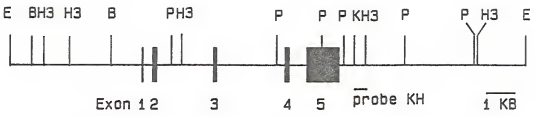
I also used the probe KH to study the chromatin structure at the 3' end of rat MnSOD gene. This probe abuts the KpnI restriction site at the 3' end of rat MnSOD gene and recognizes a 10 Kb KpnI DNA fragment located downstream from the gene, as shown in figure 4-7, lane 0. When genomic DNA isolated from DNase I-treated nuclei was digested with the restriction endonuclease, KpnI, fractionated on a 1% agarose gel, electrotransferred to a nylon membrane, and hybridized with probe KH, no strong DNase I HS sites were detected within this 10 Kb region in control as well as treated cells, as shown in figure 4-7.

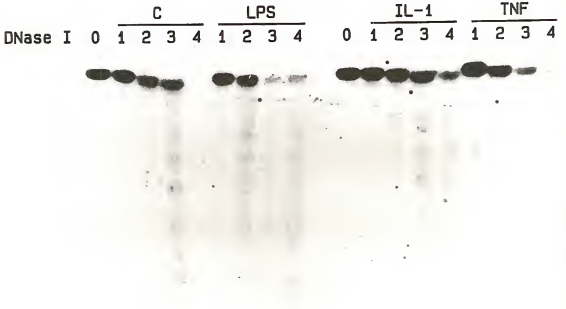
The probe BH was designed to study the chromatin structure changes at the 5' promoter region of rat MnSOD gene. Following nuclear isolation and DNase I treatment, the purified genomic DNA was digested with the restriction

Figure 4-7. DNase I hypersensitive sites in rat MnSOD gene (KpnI and probe KH).

Genomic DNA was obtained as described in figure 4-5 and the DNase I concentrations used were 0: 0 U/ml, 1: 100 U/ml, 2: 150 U/ml, 3: 200 U/ml and 4: 300 U/ml. Ten μg of genomic DNA was digested with KpnI, fractionated on 1% agarose gel, electrotransferred, and probe KH was used to reveal the DNase I HS sites.

The restriction map of rat MnSOD gene shown above illustrates the relative position of the probe used. In addition, the approximate scale of 1 kb is shown. However, no HS site was detected in either control or treated cells.


E BH3 H3 B PH3 P P P KH3 P P H3 E
Exon 1 2 3 4 5
probe KH
1 KB


C
LPS
IL-1
TNF
DNase I 0 1 2 3 4 1 2 3 4 0 1 2 3 4 1 2 3 4

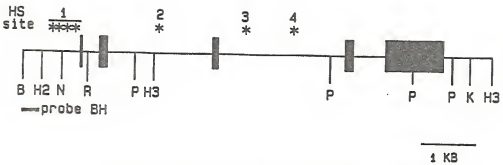
endonuclease, BamHI, fractionated on a 1% agarose gel, electrotransferred to a nylon membrane, and hybridized with probe BH. As shown in figure 4-8, lane 0, this probe recognized a 15 Kb genomic BamHI fragment in the nuclei treated with no DNase I. In control cells treated with increasing concentrations of DNase I, this probe detected 4 HS sites, specifically sites 1 to 4. This Southern analysis also increased the resolution of HS site 1, revealing the presence of four HS subsites, a, b, c, and d, in this region. Because of the unusual high frequency of DNase I digestion around site 1, shown by the strong intensity of HS site 1, the signals for the other HS sites are diminished depending on the direction of detection. In LPS, IL-1, and TNF treated cells, with increasing concentrations of DNase I, the same four HS sites were observed. However, in the treated cells, subsite c of HS site 1 showed a change in DNase I digestion frequency. To further investigate the possible changes in stimulus-dependent chromatin structure occurred in the promoter region of rat MnSOD gene, a 4% native polyacrylamide gel and a 2.5% Metaphor agarose gel were used to enhance the resolution of the region of interest, which will be discussed in detail in chapter 5.

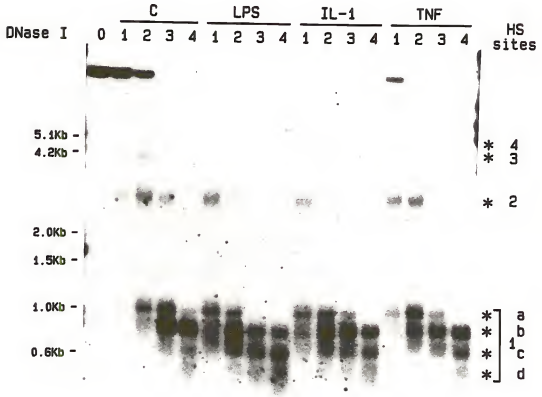
I was also concerned that the strong HS digestion in site 1 caused by the unusual high frequency of DNase I activity might interfere with the detection of weaker HS sites existing further 5′ from the end of the HS site 1.

Figure 4-8. DNase I hypersensitive sites in rat MnSOD gene (BamHI and probe BH).

Genomic DNA was obtained as described in figure 4-5 and the DNase I concentrations used were 0: 0 U/ml, 1: 75 U/ml, 2: 100 U/ml, 3: 150 U/ml, and 4: 200 U/ml. Ten μg of genomic DNA was restricted with BamHI, fractionated on 1% agarose gel, electrotransferred, and probe BH was used to reveal the DNase I HS sites.

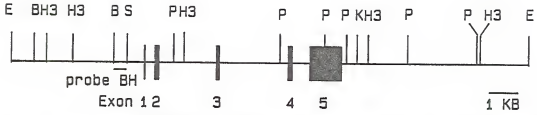
The restriction map of rat MnSOD gene shown above illustrates the relative positions of the probe used and the HS sites revealed.

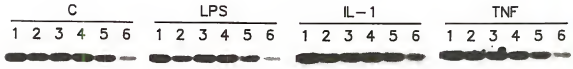

HS
site
1
2
3
4
B H2 N R P H3
P
P
P K H3
probe BH
1 KB


DNase I
C
LPS
IL-1
TNF
0 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
HS
sites
5.1Kb
4.2Kb
2.0Kb
1.5Kb
1.0Kb
0.6Kb
4
3
2
a
b
1
c
d

Because probe BH is about 76 bp 5′ from a SspI site existing at the 5′ promoter region of rat MnSOD gene and recognizes a 8 Kb SspI DNA fragment upstream the promoter region, this is also a suitable probe for further study of the 5′ end of rat MnSOD gene. The genomic DNA isolated from DNase I-treated nuclei was digested with SspI, fractionated on a 1% agarose gel, electrotransferred to a nylon membrane, and hybridized with probe BH. No DNase I HS site has been identified in this 8 Kb upstream region in either control or LPS, IL-1, and TNF treated cells, as shown in figure 4-9. Combined with the results shown in figure 4-8, I have concluded that HS site 1 is the only HS site identified within 8 Kb of the 5′ promoter region of the rat MnSOD gene. Interestingly, this HS site is also the strongest one compared to the other HS sites detected. More importantly, the intensity change which occurred within this site when L2 cells were treated with LPS, IL-1, and TNF implies a possible change in chromatin structure caused by the binding or removal of a transcription factor(s) in the promoter region, which will be further investigated in chapter 5.

In summary, as shown in figure 4-10, DNase I hypersensitivity studies identify seven HS sites along the rat MnSOD gene. These HS sites are located from about 0.7 Kb upstream from the transcription initiation site to the end of this gene. A total of 25 Kb of the genome around the rat MnSOD gene has been evaluated for DNase I


E BH3 H3 B S PH3 P P P KH3 P P H3 E
probe BH
Exon 1 2 3 4 5
1 KB


C
LPS
IL-1
TNF
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6

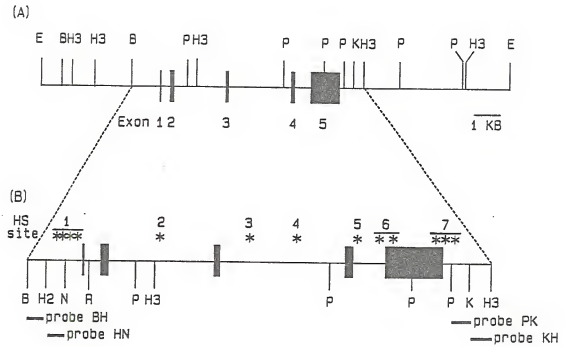


Figure 4-10. DNase I hypersensitive sites in rat MnSOD gene.
The composite DNase I hypersensitive sites map along with rat MnSOD genomic map is shown.
(A) Complete restriction map of rat MnSOD gene. The five exons of MnSOD gene are shown as black rectangles. The code for the restriction enzymes appears at the bottom of the figure and the approximate scale is designated by the 1 Kb marker on the left.
(B) Map of DNase I hypersensitive sites. The region encompassing the five exons is expanded and the location of the DNase I hypersensitive (HS) sites are designated by * and numbered 1-7. The four dark lines below the expanded map illustrate the position of the four probes used: probe BH (BamHI to HincII); probe HN (HincII to NcoI); probe PK (PstI to KpnI); and probe KH (KpnI to HindIII).

hypersensitivity, no DNase I HS sites have been detected beyond the promoter region or the 3' end of the gene. Upon treatment with LPS, IL-1, or TNF, the same seven HS sites were observed except that the intensity of the subsite c within HS site 1 changed, indicating a change of DNase I digestion frequency. In chapter 5, I will further investigated this region using a high resolution electrophoresis system.

CHAPTER 5
HIGH RESOLUTION CHROMATIN STRUCTURE ANALYSIS OF RAT MANGANESE SUPEROXIDE DISMUTASE GENE

Introduction

In chapter 4, I have described that a total of seven HS sites have been identified along the rat MnSOD gene using DNase I hypersensitivity studies. I have also observed a change in intensity within subsite c of HS site 1 upon the cellular treatment of LPS, IL-1, and TNF. However, because of the limitation in resolution inherent to traditional agarose gel methodology, I was unable to further define any detailed changes in chromatin structure associated with the promoter region of rat MnSOD gene.

Pauli et al. (1988) have developed an approach to greatly increase the resolution of mapping HS sites. In this protocol, after nuclear isolation and DNase I treatment, genomic DNA was purified, digested with a restriction endonuclease, fractionated on a 4% native polyacrylamide gel, followed by electrotransfer and Southern analysis. The increased resolution of the polyacrylamide gel enabled them to scan an area of several hundred base pairs 5' to human histone H4 gene with an accuracy of +/- 5-10 base pairs. Protein-DNA interactions were seen on the

autoradiograph as light areas and DNase I HS sites as dark bands. This method, therefore, provides a rapid and relatively simple way to accurately localize protein-DNA interactions as well as DNase I HS sites in the region of interest on the same autoradiograph.

In this chapter, I have applied the polyacrylamide gel electrophoresis system to further characterize the promoter region of rat MnSOD gene. However, because the 4% polyacrylamide gel is very soft, sticky, and unmanageable, it was difficult to handle and manipulate. I have, therefore, also developed another gel electrophoresis system. Using 2.5% Metaphor agarose gel, I have been able to achieve the resolution similar to that obtained with 4% polyacrylamide gel, but with the ease of a traditional agarose gel.

In addition, I have also reevaluated my nuclear run-on and DNase I hypersensitivity studies in lysolecithin-permeabilized cells using another methodology which I have perfected for these studies. To enable the entry of nucleotide triphosphate and DNase I into cells, the nuclei were traditionally isolated and nuclear run-on or DNase I hypersensitivity studies were performed. However, nuclei often were found to be deficient in initiation by RNA polymerase II. Miller et al. (1978) first evaluated lysolecithin-permeabilized cells and found that these cells retained their general morphology as well as intact

organelles. These permeabilized cells were able to synthesize protein, RNA, and DNA when supplied with appropriate substrates and cofactors, many of which do not penetrate intact cells. Therefore, the lysolecithin-permeabilized cells are useful for studying nascent RNA synthesis and chromatin structure under circumstances approaching physiological conditions. Pfeifer & Riggs (1991) recently reported that several footprints in the promoter region of human phosphoglycerate kinase gene were observed in lysolecithin-permeabilized cells but not in isolated nuclei. Since lysolecithin permeabilization is a milder approach than the rather unphysiological procedure of nuclear preparation, it might avoid the possible loss of transcription factors and provide a transcription system which better approximates the in vivo situation.

Results

Protein-DNA Interactions in the Promoter Region of Rat MnSOD Gene in Isolated Nuclei

To further characterize the intensity change of HS site 1 in the promoter region of rat MnSOD gene upon the treatment of LPS, IL-1, and TNF, a 4% polyacrylamide gel was employed to reveal a more detailed view of the chromatin structure within this region. After L2 cells were treated with 0.5 μg/ml of LPS, nuclei were isolated and incubated with increasing concentrations of DNase I at 37°C for 4 minutes. The genomic DNA was isolated, digested with the

restriction endonucleases, HincII and RsaI, fractionated on a 4% native polyacrylamide gel, and hybridized with probe HN. As shown in figure 5-1 lane 0, this probe recognized a 0.95 Kb HincII-RsaI DNA fragment in the genome of nuclei treated with no DNase I. In control cells treated with increasing concentrations of DNase I, four discrete DNase I-resistant regions, as light areas on the autoradiograph, were found. These DNase I-resistant regions have been designated as protein binding (PB) sites 1 to 4 and we believe these regions are in fact protein footprints flanked by strong HS sites.

These four PB sites have also been observed in LPS-treated cells, indicating that these four PB sites are probably involved in the basal expression of rat MnSOD gene. In addition, an inducible HS site, shown as a * in figure 5-1, was only found in LPS-treated cells. This inducible HS site created an extra DNase I-resistant region, shown as PB site 5 in figure 5-1, indicating that an additional transcription factor(s) involved in the regulation of MnSOD gene by LPS interacted with a cis-acting element in this region.

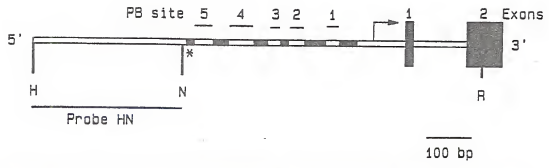
Transcriptional Regulation of Rat MnSOD Gene in Permeabilized Cells

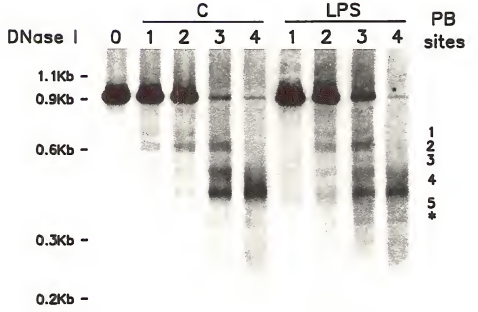
To reevaluate the nuclear run-on studies in lysolecithin-permeabilized cells, L2 cells were treated with 0.5 μg/ml LPS for 1 hour, and permeabilized by incubating with 0.05% lysolecithin for 1 minute. [α-^{32}P]-UTP and

Figure 5-1. Protein-DNA interaction in rat MnSOD promoter region revealed by 4% polyacrylamide gel (LPS treatment).

After L2 cells were treated with 0.5 μg/ml LPS for 4 hours, nuclei were isolated and treated with increasing concentrations of DNase I (0: 0 U/ml, 1: 75 U/ml, 2: 100 U/ml, 3: 150 U/ml, and 4: 200 U/ml) at 37°C for 4 minutes. Twenty μg of purified genomic DNA was restricted with HincII and RsaI, and subjected to 4% native polyacrylamide gel electrophoresis. After electrotransfer, the DNase I-resistant regions, which have been shown as possible protein binding (PB) sites, were revealed by probe HN. At least 4 protein binding sites were identified in control cells; and 5, in treated cells. In addition, the extra HS site observed in treated cells is designated as *.

The restriction map shown above indicates the probe used and the relative positions of the protein binding sites revealed. The transcription initiation site is shown as an arrow. The codes for restriction enzymes are: H, HincII, N: NcoI, and R: RsaI. The approximate scale for 100 bp size marker is also shown.


PB site 5 4 3 2 1
1
2 Exons
5'
3'
*
H
N
R
Probe HN
100 bp


C
LPS
PB sites
DNase I
0 1 2 3 4 1 2 3 4
1.1Kb –
0.9Kb –
0.6Kb –
0.3Kb –
0.2Kb –
1
2
3
4
5
*

nucleotides were added to the permeabilized cells and the transcription allowed to continued at 30°C for 15 minutes. Total RNA, including nascent radioactive RNA, was isolated by acid GTC-phenol chloroform extraction method and hybridized with dot blots containing DNA fragments of rat MnSOD cDNA, rat Cu/ZnSOD cDNA, mouse actin cDNA, and linearized plasmid pUC19. After washing, the blots were subjected to autoradiography and the change of transcription rate of rat MnSOD gene was calculated by densitometry.

As shown in figure 5-2, the transcription rate of MnSOD gene increased to 9.7 fold after 1 hour LPS treatment using actin as an internal control. The increase in the transcription rate of MnSOD gene after LPS treatment indicates that the rat MnSOD gene is at least partially transcriptionally regulated by LPS.

A comparison of the results obtained from the permeabilized cells versus the 3.1 fold increase in the transcriptional rate found previously in nuclei clearly indicates that permeabilized cells are probably a more suitable system for evaluation of transcriptional regulation and therefore DNase I hypersensitivity studies.

Protein-DNA Interactions in the Promoter Region of Rat MnSOD Gene in Permeabilized Cells

Since the results obtained from the nuclear run-on studies indicated that the permeabilized cells are a better system for evaluating gene regulation in vivo, the DNase I hypersensitivity studies were repeated in the permeabilized

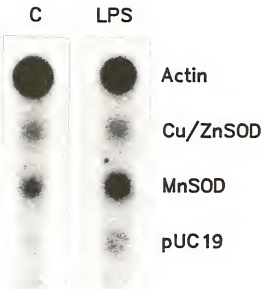


Figure 5-2. Nascent RNA synthesis in permeabilized cells.
L2 cells were treated with 0.5 μg/ml LPS for 1 hour, and permeabilized with 0.05% lysolecithin. Nascent RNA synthesis were performed at 30°C for 15 minutes. Total RNA, including the radioactive nascent RNA, were isolated and hybridized with the dot blots containing the DNA fragments of actin, Cu/ZnSOD, MnSOD, and linearized pUC19 plasmid. After wash, the membranes were exposed to x-ray film with intensifying screen at -85°C for 1 week.

cells to further confirm the inducible HS site observed in the isolated nuclei from the cells treated with LPS. Also, a new gel system, 2.5% Metaphor agarose gel in TAE buffer, was used to achieve a similar resolution and a more manageable system compared to the 4% native polyacrylamide gel.

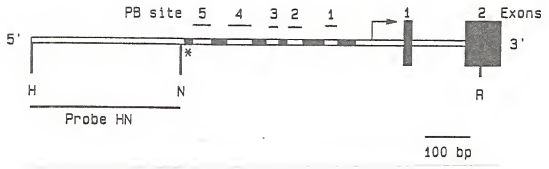
L2 cells treated with 0.5 μg/ml LPS for 4 hours were permeabilized by incubating with 0.05% lysolecithin for 1 minute. The permeabilized cells were incubated with increasing concentrations of DNase I, and the purified genomic DNA was digested with restriction endonucleases, RsaI and HincII. The DNA was fractionated on a 2.5% Metaphor agarose gel, and electrotransferred to a noncharged nylon membrane. The membrane was hybridized with probe HN and subjected to autoradiography.

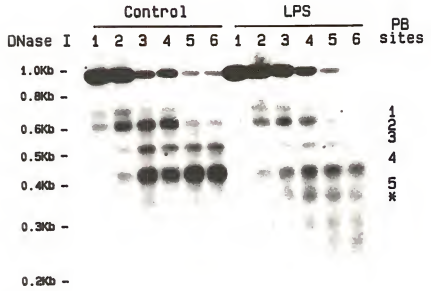
As shown in figure 5-3, in control cells, four discrete DNase I-resistant regions, indicated as PB sites 1-4, were found. These four protein footprints pattern were also observed in cells treated with LPS for 4 hours, indicating that these four PB sites are probably involved in the basal expression of rat MnSOD gene. More importantly, an inducible HS site, shown as * in figure 5-3, was found only in LPS-treated cells. This extra HS site indicates that an additional transcriptional factor(s), shown as PB site 5 in figure 5-3, involved in the regulation of MnSOD gene by LPS interacts with a cis-acting element(s) in this region.

Figure 5-3. Protein-DNA interaction in rat MnSOD promoter region revealed by 2.5% Metaphor agarose gel (LPS treatment).

L2 cells were treated with 0.5 μg/ml LPS for 4 hours, permeabilized with 0.05% lysolecithin, and treated with increasing concentrations of DNase I (1: 133 U/ml, 2: 156 U/ml, 3: 185 U/ml, 4: 213 U/ml, 5: 240U/ml, and 6: 267 U/ml) at 37°C for 4 minutes. Fifteen μg of purified genomic DNA was restricted with HincII and RsaI, and fractionated on a 2.5% Metaphor agarose gel. After electrotransfer, the DNase I-resistant regions, which have been shown as possible protein binding (PB) sites, were revealed by probe HN. At least 4 protein binding sites were identified in control cells; and 5, in treated cells. In addition, the extra HS site observed in treated cells is designated as *.

The restriction map shown above indicates the probe used and the relative positions of the protein binding sites revealed. The transcription initiation site is shown as an arrow. The codes for restriction enzymes are: H, HincII, N: NcoI, and R: RsaI. The approximate scale for 100 bp size marker is also shown.


PB site 5 4 3 2 1
1
2 Exons
5'
3'
*
H
N
R
Probe HN
100 bp


Control
LPS
DNase I 1 2 3 4 5 6 1 2 3 4 5 6
PB sites
1.0Kb -
0.8Kb -
0.6Kb -
0.5Kb -
0.4Kb -
0.3Kb -
0.2Kb -
1
2
3
4
5
*

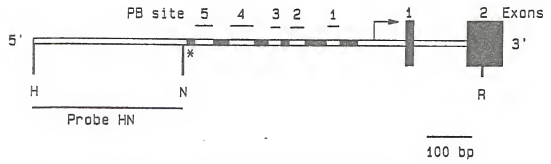
The possibility for a change in chromatin structure following IL-1α treatment was also evaluated. After L2 cells were treated with 2 ng/ml IL-1α for 4 hours, the cells were permeabilized and treated with increasing concentrations of DNase I. Fifteen μg of purified genomic DNA were restricted with HincII and RsaI, fractionated on a 2,5% Metaphor agarose gel, electrotransferred, and hybridized with probe HN. As shown in figure 5-4, four DNase I-resistant regions, shown as PB sites 1-4, were detected in control cells. The same protein footprint pattern was detected in IL-1 treated cells except an inducible HS site, shown as * in figure 5-4, indicating an additional transcription factor(s) were added to the basal transcriptional machinery.

In figure 5-5, the chromatin structural change induced by TNF treatment was also investigated. L2 cells treated with 10 ng/ml TNF for 4 hours were permeabilized and treated with increasing concentrations of DNase I. The purified genomic DNA was digested with HincII and RsaI, fractionated on a 2.5% Metaphor agarose gel, electrotransferred, and hybridized with probe HN. Similar results were obtained from TNF-treated cells as compared to LPS- or IL-1-treated cells. Four DNase I-resistant regions, shown as PB sites 1-4 in figure 5-5, were found in both control and TNF-treated cells. Upon TNF treatment, an inducible HS site, indicated as * in figure 5-5, was only found in treated, but not

Figure 5-4. Protein-DNA interaction in rat MnSOD promoter region revealed by 2.5% Metaphor agarose gel (IL-1 treatment).

L2 cells were treated with 2 ng/ml IL-1 for 4 hours, permeabilized with 0.05% lysolecithin, and treated with increasing concentrations of DNase I (1: 133 U/ml, 2: 156 U/ml, 3: 185 U/ml, 4: 213 U/ml, 5: 240U/ml, and 6: 267 U/ml) at 37°C for 4 minutes. Fifteen μg of purified genomic DNA was restricted with HincII and RsaI, and fractionated on a 2.5% Metaphor agarose gel. After electrotransfer, the DNase I-resistant regions, which have been shown as possible protein binding (PB) sites, were revealed by probe HN. At least 4 protein binding sites were identified in control cells; and 5, in treated cells. In addition, the extra HS site observed in treated cells is designated as *.

The restriction map shown above indicates the probe used and the relative positions of the protein binding sites revealed. The transcription initiation site is shown as an arrow. The codes for restriction enzymes are: H, HincII, N: NcoI, and R: RsaI. The approximate scale for 100 bp size marker is also shown.


PB site 5 4 3 2 1
1
2 Exons
5'
3'
*
H
N
R
Probe HN
100 bp

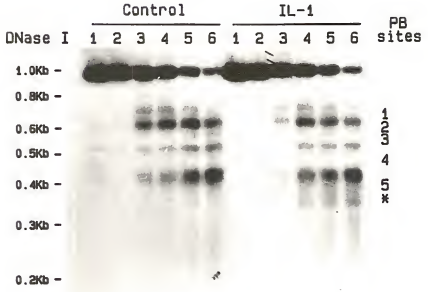
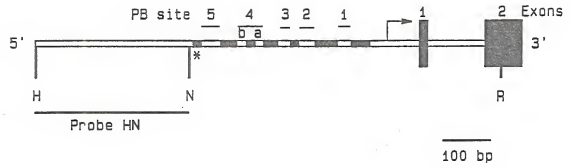
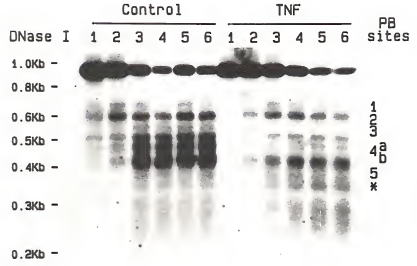

Control
IL-1
DNase I 1 2 3 4 5 6 1 2 3 4 5 6
PB sites
1.0Kb
0.8Kb
0.6Kb
0.5Kb
0.4Kb
0.3Kb
0.2Kb
1
2
3
4
5
*

Figure 5-5. Protein-DNA interaction in rat MnSOD promoter region revealed by 2.5% Metaphor agarose gel (TNF treatment).

L2 cells were treated with 10 ng/ml TNF for 4 hours, permeabilized with 0.05% lysolecithin, and treated with increasing concentrations of DNase I (1: 133 U/ml, 2: 156 U/ml, 3: 185 U/ml, 4: 213 U/ml, 5: 240U/ml, and 6: 267 U/ml) at 37°C for 4 minutes. Fifteen μg of purified genomic DNA was restricted with HincII and RsaI, and fractionated on a 2.5% Metaphor agarose gel. After electrotransfer, the DNase I-resistant regions, which have been shown as possible protein binding (PB) sites, were revealed by probe HN. At least 4 protein binding sites were identified in control cells; and 5, in treated cells. In addition, the extra HS site observed in treated cells is designated as *.

The restriction map shown above indicates the probe used and the relative positions of the protein binding sites revealed. The transcription initiation site is shown as an arrow. The codes for restriction enzymes are: H, HincII, N: NcoI, and R: RsaI. The approximate scale for 100 bp size marker is also shown.


PB site 5 4 3 2 1
b a
1
2 Exons
5'
3'
*
H
N
R
Probe HN
100 bp


Control
TNF
DNase I 1 2 3 4 5 6 1 2 3 4 5 6
PB sites
1.0Kb -
0.8Kb -
0.6Kb -
0.5Kb -
0.4Kb -
0.3Kb -
0.2Kb -
1
2
3
4a
b
5
*

control cells. Interestingly, an extra HS site located within PB site 4 was occasionally observed in both control and treated cells, as shown in figure 5-5. This extra HS site has been observed on more than one occasion in both control and treated cells, indicating that this extra HS site is not involved in the regulation of MnSOD by LPS, IL-1, and TNF, but rather is an alteration in chromatin structure associated with basal expression. To accommodate this extra HS site observed occasionally, PB site 4 is further delineated to PB site 4a and 4b.

A proposed model for rat MnSOD gene regulation is presented in figure 5-6 which combines the results obtained from figures 5-1, 5-3, 5-4, and 5-5. In control cells, four transcriptional complexes interact with the promoter region for the basal expression of rat MnSOD gene. Upon stimulant treatment, an additional transcription factor(s) is found to interact with the MnSOD promoter region, as demonstrated by the inducible HS site observed only in the treated cells. The interaction of this transcription factor(s) with the cis-acting element(s) in the promoter region increases the transcriptional rate of the rat MnSOD gene, therefore, increasing the steady-state levels of MnSOD mRNA.

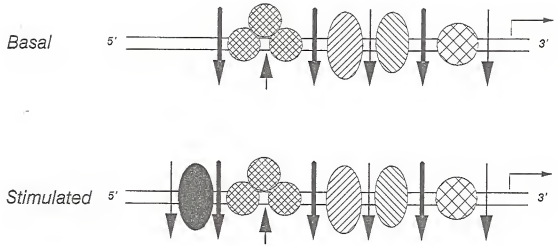


Figure 5-6. The occupancy of basal and stimulated MnSOD promoter.

A model for the regulation of rat MnSOD gene expression by LPS, IL-1, and TNF is presented according to the results obtained from the DNase I hypersensitivity studies. In basal expression, at least four transcription factor complexes interact with rat MnSOD promoter region. These four transcription factor complexes are flanked by DNase I hypersensitive sites, indicating by the arrows. However, since two different patterns of DNase I HS sites in transcription factor complex 4 have been observed, an extra arrow is added to show the extra HS site observed occasionally. In stimulated cells, an extra HS site in the 5' end of this region has been observed, indicating an additional transcription factor complex interacts with the promoter region, which consequently contributes to the increased expression of rat MnSOD gene.

Discussion

Transcriptional control in eukaryotes results from an interplay between regulatory DNA sequences and site-specific DNA binding proteins. Many transcription factors, which bind to their own specific DNA sequences, have been identified. Several transcription factors which may be involved in the regulation of MnSOD gene by LPS, IL-1, and TNF are discussed.

The nuclear factor kappa B (NF-κB) is a transcription factor which has been widely shown to be associated with gene regulation by LPS, IL-1, and TNF (reviews in Blank et al., 1992; Grimm & Baeuerle, 1993). NF-κB was first characterized as a factor binding to a sequence motif (κB) located in the immunoglobulin κ light chain enhancer gene (Sen & Baltimore, 1986). Subsequently it was shown to be involved in the regulation of several other cellular and viral genes, such as the regulation of IL-2 receptor α-chain gene expression by TNF (Lowenthal et al., 1989). Various agents are known to activate this factor, including mitogens (phorbol esters, lectins), cytokines (IL-1, TNF), and LPS. An inactive cytoplasmic form of NF-κB contains three subunits: p50, p65, and IκB, an inhibitory protein. Cytoplasmic NF-κB can be activated by releasing inhibitory protein IκB after protein kinase C-dependent phosphorylation (Beg et al., 1993). Activated NF-κB which consists of p50 and p65 is consequently translocated to the nucleus, and

this heterodimer interacts with DNA by binding to κB motifs in promoters and enhancers (Urban et al., 1991). Both p50 and p65 belong to the rel gene family which contains a region referred to as the NF-κB/Rel/dorsal (NDR) domain which is required for DNA binding and dimerization of the proteins (Blank et al., 1992). Mature p50 is generated by processing a precursor protein, p105, through an ATP-dependent pathway (Fan , 1991).

Another possible nuclear mediator for the actions of IL-1 and TNF is AP-1 transcription factor. It has been shown that an IL-1 responsive element in the promotor region of human IL-2 gene is similar to the binding site for the transcription factor AP-1 (Muegge et al., 1989). It has also been demonstrated that stimulation of collagenase gene transcription by TNF is mediated by a cis-acting element that is responsive to the transcription factor AP-1 (Brenner et al., 1989). The sequence that is vital for AP-1 factor binding is TGACTCA (Lee et al., 1987). It has also been shown that SP-1 transcription factor may play an important role in the gene regulation by TNF.

Several potential regulatory elements for controlling the transcription of rat MnSOD gene have been located in the promoter region. A consensus sequence for the AP-1 enhancer binding protein has been found in the 5′ promoter region of the rat MnSOD gene at the position of -434 to -427. These cis-acting elements are known to be recognized by variable

protein dimers formed by members of the Jun and Fos families. Also, two copies of the consensus sequence for the SP-1 binding protein have been identified in the promoter region of rat MnSOD gene at the positions of -14 to -9 and -238 to -233. A putative NF-κB binding site, GGGGCTTTCC, has also been found at the position of -359 to -350 in the promoter region of rat MnSOD gene by comparison with the consensus sequences, GGGRNTYYCC (R: purines, N: nucleotides, Y: pyrimidines) (Sen & Baltimore, 1986).

The data obtained in figure 5-1, 5-3, 5-4, and 5-5 suggest that a transcription factor(s) interacting with the PB site 5 may be responsible for the regulation of rat MnSOD gene by LPS, IL-1, and TNF. The extra DNase I HS site flanking PB site 5 strongly indicates that a transcriptional factor(s) involved in the regulation of MnSOD gene is added to this region. We have roughly localized the PB site 5 in the promoter region of the rat MnSOD gene to the position of -365 to -340 relative to the transcription initiation site. Since NF-κB protein has been widely shown to be involved in LPS, IL-1, and TNF response and the potential NF-κB binding site is located in the promoter region of rat MnSOD gene at the position of -359 to -350, it is possible that the interaction of NF-κB with the promoter region of MnSOD gene contributes to the extra HS site flanking PB site 5 after LPS, IL-1, and TNF treatment. When L2 cells were co-treated with cycloheximide and LPS, IL-1, or TNF, the induction of

rat MnSOD mRNA was not abolished, but increased, indicating that no protein synthesis is required for the regulation of MnSOD gene by these inflammatory mediators. Since the activation of NF-κB is also enhanced by cycloheximide treatment (Sen & Baltimore, 1986), the cycloheximide data further support the involvement of NF-κB in the regulation of rat MnSOD gene by LPS, IL-1, and TNF. However, more detailed *in vivo* footprinting will be conducted to further explore the possibility of the involvement of NF-κB or any other transcription factors in the regulation of rat MnSOD gene expression.

Since at least four PB sites flanked by constitutive HS sites have been found in the promoter region of rat MnSOD gene in both control and treated cells, the involvement of transcription factors in basal transcriptional machinery is also discussed. There are three eukaryotic nuclear RNA polymerases, each with a distinct biological function: RNA polymerase I synthesizes ribosomal RNA, RNA polymerase II synthesizes messenger RNA and several small nuclear RNAs, and RNA polymerase III synthesizes transfer RNA, 5s rRNA, and various other small cellular and viral RNAs. The cis-acting sequences necessary for accurate and efficient initiation in the basal transcriptional machinery for the RNA polymerase II promoter are upstream of the transcribed region. These comprise an initiator element at the transcription start site, a TATA box around -30, and the

CCAAT box around -60. The TATA sequence, a critical element for initiation by RNA polymerase II, is recognized by a sequence specific TATA-binding protein (TBP) (Rigby, 1993), which is a component of a large complex of proteins forming the basal transcription factor TFIID. In higher organisms native TFIID is a large, heterogeneous, multisubunit complex composed of TBP and a variety of tightly bound TBP-associated factors (TAFs) (Pugh et al., 1992). After initial binding of TFIID to the TATA box, TFIIB interacts with the TBP and in association with TFIIF, RNA polymerase II next binds to the preinitiation complex (Hahn, 1993).

Less is known about how initiation of transcription is directed on promoters lacking TATA elements. TBP has long been regarded as a factor required by those class II genes with TATA boxes. Recent evidence has suggested that TBP and TAFs are also involved in recognizing TATA-less promoter sequences. Studies have shown that artificial template constructs lacking TATA sequences still require TFIID fractions to reconstitute transcription by RNA polymerase II. For promoters that do not contain the TATA element, sequences that flank the site of initiation have been implicated as important elements for mediating the stable association of the TBP with the promoter. Wiley et al. (1992) have demonstrated that many TATA-less promoters contain important sequence elements 30 bp upstream of their transcription initiation site which interact with TFIID and

direct RNA polymerase II to initiate transcription. In addition, Sp1 has also been implicated in the transcription of TATA-less promoters (Faber et al., 1993).

No TATA box or CCAAT box has been found in the nucleotide sequence upstream from the transcription initiation site of rat MnSOD gene. Since at least four potential protein binding sites have been identified in the promoter region of rat MnSOD gene, it is very likely that these protein binding sites interact with the transcription factors required for the basal expression of this TATA-less gene. Further study of the protein-DNA interactions in the promoter region of rat MnSOD gene might help to clarify the possible role of TBP in TATA-less transcription as well as identify the cis-element sequences required for TATA-less transcription.

CHAPTER 6
CONCLUSIONS AND FURTHER DIRECTIONS

SODs which dismutate superoxide into hydrogen peroxide and water are thought to play a vital role in protecting cells from free radical damage. Free radical damage has been associated with various diseases and aging. The studies of the gene structure and the regulation of SODs are, therefore, important.

In chapter 3, I report the purification and characterization of several rat Cu/ZnSOD cDNA clones from a rat cDNA library. The longest clone, c14-2, contains 75 bp of 5' noncoding sequence, 462 bp of coding sequence corresponding to 154 amino acids, and 91 bp of 3' non-coding sequence, for a total of 628 bp.

In addition, a 13 kb rat Cu/ZnSOD genomic clone, G1-1, has been purified from a rat liver genomic library and completely characterized by restriction mapping, detailed sequencing and Southern blot analysis. This gene spans approximately 6 kb and contains five exons and four introns.

Comparison of rat, mouse, and human Cu/ZnSOD genes reveals a high conservation in genomic organization and exon-intron junctions, including an unusual 5'GC donor sequence at the first intron. The gene contains a TATA box

as well as an inverted CCAAT box, a feature common to both the mouse and human genes. Furthermore, several repeats were identified in the 5' promoter region of this gene, and these regulatory elements are also strikingly conserved in these three species.

Recently, researchers have linked the mutation of Cu/ZnSOD gene to a hereditary form of a motor neuron disease, amyotrophic lateral sclerosis (ALS) (Rosen et al., 1993; Deng et al., 1993). The isolation and characterization of rat Cu/ZnSOD gene is, therefore, important for further understanding the expression and regulation of this gene and the diseases associated with it.

In studies reported in chapter 4, I investigated the changes in chromatin structure associated with the regulation of rat MnSOD gene by the inflammatory mediators, LPS, IL-1, and TNF. It has been previously demonstrated that the rat MnSOD, but not Cu/ZnSOD, steady-state mRNA levels increased upon cellular treatment with LPS, IL-1, or TNF. I have performed nuclear run-on studies to illustrate that the increase in rat MnSOD mRNA level by these inflammatory mediators is in part due to transcriptional regulation. However, the increase in the transcriptional rate measured in nuclei or permeabilized cells may not account for the total increase in mRNA steady-state level, therefore, mRNA stabilization is proposed as an additional regulatory mechanism for the regulation of rat MnSOD by LPS,

IL-1, and TNF. The possible involvement of mRNA stabilizing element(s) (Sachs, 1993) will be investigated in the future using deletion analysis.

I have also evaluated the changes in chromatin structure in rat MnSOD gene by LPS, IL-1, and TNF using DNase I hypersensitivity studies. A total of seven hypersensitive sites have been identified along the rat MnSOD gene. The strongest HS site is HS site 1, which is located in the promoter region of rat MnSOD gene and has shown difference in subsite intensity in control versus treated cells, indicating this region is involved in the regulation of rat MnSOD gene.

However, the significance of the other six HS sites located in the regions other than the promoter region are not clear. Recent evidence has suggested that sequences critical to the regulation of the MnSOD may exist in HS sites other than those found in the promoter region. An androgen-inducible HS site has been found in the proximal end of intron 9 of the murine β-glucuronidase gene and the protein-DNA interaction in this inducible HS site has been indicated by gel shift assay (Lund et al., 1991). A constitutive DNase I HS site in the first intron of the alpha 1 collagen gene has been demonstrated containing multiple protein-DNA binding sites (Liska et al., 1992). In addition, a retinoic acid responsive element exists in a

3' DNase I HS site of a homeobox gene (Langston & Gudas, 1992).

HS sites detected by DNase I hypersensitivity studies provide information about physical characteristics of chromatin structure, however, they do not provide information about the functional roles of each HS site detected. Further analysis of the HS sites identified along the rat MnSOD gene by deletion analysis in transfected cells will provide evidence for the possible roles, such as enhancers, or mRNA stabilizing elements, of each HS site. Moreover, a detailed DNA-protein footprint at the nucleotide level resolution should reveal the fine architecture of each HS site.

A total of 25 Kb of rat MnSOD genome has been evaluated for DNase I hypersensitivity and no HS site was observed 8 kb upstream of the promoter region of rat MnSOD gene except HS site 1. However, because of the limit of the availability of genomic clones, no further region has been investigated. Cis-acting DNA elements that sometimes mediate position-independent expression of genes over a long distance such as a locus control region (LCR) have been reported. An LCR was first demonstrated in human β-globin gene. In the chromatin of human β-globin gene cluster, the LCR was marked by a set of four strong DNase I HS sites located 10-20 kb upstream of the ϵ-globin gene (Tuan et al., 1985). The region has been shown to control the β-globin

locus over a distance of at least 150 kb (Grosveld et al., 1987). Therefore, the possibility of the involvement of LCR in the regulation of rat MnSOD gene may need to be further explored.

In studies reported in chapter 5, the HS site 1 was further investigated by high resolution analysis of chromatin structure using a 4% native polyacrylamide gel or 2.5% metaphor agarose gel. This HS site was found to contain at least 4 discrete DNase I-resistant regions in control cells. Based on previous studies (Pauli et al., 1988), the resistant regions are believed to be sites for protein footprints flanked by strong HS sites. In addition, the stimulated cells contain an inducible HS site in this 5' region, demonstrating that a transcriptional factor(s) involved in the regulation of rat MnSOD gene by LPS, IL-1, and TNF is added to this region.

This inducible HS site detected in the cells treated with LPS, IL-1, or TNF will be further examined by genomic sequencing. This technique will reveal any differences at the nucleotide level and, therefore, the exact promoter sequences involved in the regulation of rat MnSOD gene by these inflammatory mediators will be delineated. The information obtained from chapters 4 and 5 will help to define a rather small region for in vivo genomic sequencing.

At least four potential protein binding sites that may be involved in the basal transcriptional machinery have been

identified in the promoter region of rat MnSOD gene. Since no TATA box or CCAAT box has been found in the nucleotide sequence upstream from the transcription initiation site of rat MnSOD gene, it is very likely that some of these protein binding sites interact with the transcription factors required for the basal expression of a TATA-less gene. Further study of the protein-DNA interactions in the promoter region of rat MnSOD gene using in vivo genomic sequencing might shed some light on the cis-element sequences required for TATA-less transcription.

REFERENCES

Aggarwal, B.B., Kohr, W.J., Hass, P.E., Moffat, B., Spencer, S.A., Henzel, W.J., Bringman, T.S., Nedwin, G.E., Goeddel, D.V., and Harkins, R.N. (1985). Human tumor necrosis factor. Production, purification, and characterization. J. Biol. Chem. 260, 2345-2354.

Asayama, K. and Burr, I.M. (1985). Rat superoxide dismutases. J. Biol. Chem. 260, 2212-2217.

Asayama, K., Janco, R.L., and Burr, I.M. (1985a). Selective induction of manganous superoxide dismutase in human monocytes. Am. J. Physiol. 249, c393-c397.

Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M., and Dinarello, C.A. (1984) Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proc. Natl. Acad. Sci. U.S.A. 81, 7907-7911.

Bannister, J.V., Bannister, W.J., and Rotilio, G. (1987). Aspects of the structure, function, and applications of superoxide dismutase. CRC Crit. Rev. Biochem. 22, 111-180.

Baud, L., Oudinet, J.P., Bens, M., Noe, L., Peraldi, M.N., Rondeau, E., Etienne, J., and Ardaillou, R. (1989) Production of tumor necrosis factor by rat mesangial cells in response to bacterial lipopolysaccharide. Kidney Int. 35, 1111-1118.

Becker, M.M., Wang, Z., Grossmann, G., and Becherer, K.A. (1989). Genomic footprinting in mammalian cells with ultraviolet light. Proc. Natl. Acad. Sci. U.S.A. 86, 5315-5319.

Becker, P., Renkawitz, R., and Schutz, G. (1984). Tissue-specific DNaseI hypersensitive sites in the 5'-flanking sequences of the tryptophan oxygenase and the tyrosine aminotransferase genes. EMBO J. 3, 2015-2020.

Beg, A.A., Finco, T.S., Nantermet, P.B., and Baldwin, A.S.Jr. (1993). Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. 13, 3301-3310.

Bellavite, P. (1988). The superoxide-forming enzymatic system of phagocytes. Free Radical Biol. Med. 4, 225-261.

Benedetto, M.T., Anzai, Y., and Gordon, J.W. (1991). Isolation and analysis of the mouse genomic sequence encoding Cu2+-Zn2+ superoxide dismutase. Gene 99, 191-195.

Berkow, R.L., Wang, D., Larrick, J.W., Dodson, R.W.,and Howard, T.H. (1987). Enhancement of neutrophil superoxide production by preincubation with recombinant human tumor necrosis factor. J. Immunol. 139, 3783-3791.

Beutler, B. and Cerami, A. (1989). The biology of cachectin/TNF--a primary mediator of the host response. Annu. Rev. Immunol. 7, 625-655.

Bichell, D.P., Kikuchi, K., and Rotwein, P. (1992). Growth hormone rapidly activates insulin-like growth factor I gene transcription in vivo. Mol. Endocrinol., 6, 1899-1908.

Blank, V., Kourilsky, P., and Israel, A. (1992). NF-kB and related proteins: Rel/Dorsal homologies meet ankyrin-like repeats. TIBS. 17, 135-140.

Bohinski, R.J., Huffman, J.A., Whitsett, J.A., and Lattier, D.L. (1993). Cis-active elements controlling lung cell-specific expression of human pulmonary surfactant protein B gene. J. Biol. Chem. 268, 11160-11166.

Bonifer, C., Hecht, A., Saueressig, H., Wither, D.M., and Sippel, A.E. (1991). Dynamic chromatin: the regulatory domain organization of eukaryotic gene loci. J. Cell. Biochem. 47, 99-108.

Boveris, A. (1977). Mitochonddrial generation of superoxide and hydrogen peroxide. Adv. Exp. Bio. Med. 78, 67.

Brenner, D.A., O'Hara, M., Angel, P., Chojkier, M., and Karin, M. (1989). Prolonged activation of jun and collagenase genes by tumour necrosis factor-alpha. Nature 337, 661-663.

Brown, K. and Fridovich, I. (1981). DNA strand scission by enzymically generated oxygen radicals. Arch. Biochem. Biophys. 206, 414-419.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159.

Chung, J.H. and Whiteley, M. (1993). A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. 74, 505-514.

Church, G. and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. U.S.A. 81, 1991-1995.

Clark, B.D., Collins, K.L., Gandy, M.S., Webb, A.C., and Auron, P.E. (1986). Genomic sequence for human prointerleukin 1 beta: possible evolution from a reverse transcribed prointerleukin 1 alpha gene. Nucleic Acids Res. 14, 7897-7914.

Crowder, C.M. and Merlie, J.P. (1988). Studies of acetylcholine receptor subunit gene expression: chromatin structural changes during myogenesis. Ciba Found. symp. 138, 52-70.

De Wet. J.R., Fukushima, H., Dejwi, N.N., Wilcox, E., O'Brien, J.S., and Helsinki, D.R. (1984). Chromogenic immunogenic immunodetection of human serum albumin and d-L-fucosidase in a human hepatoma cDNA expression library. DNA 3, 437-447.

Deng, H.X., Hentati, A., Tainer, J.A., Iqbal, Z., Cayabyab, A., Hung,W.Y., Getzoff, E.D., Hu, P., Herzfeldt, B., Roos, R.P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H.E., Ahmed, A., Roses, A.D., Hallewell, R.A., Pericak-Vance, M.A., and Siddique, T. (1993). Amyotrophic lateral sclerosis and structural defects in Cu, Zn superoxide dismutase. Science 261, 1047-1051.

Dinarello, C.A. (1992). The biology of interleukin-1. Chem. Immunol. 51, 1-32.

Dougall, W.C. and Nick, H.S. (1991). Manganese superoxide dismutase: a hepatic acute phase protein regualted by interleukin-6 and glucocorticoids. Endocrinol. 129, 2376-2384.

Dower, S.K., Sims, J.E., Cerretti, D.P., and Bird, T.A. (1992). The interleukin-1 system: receptors, ligands and signals. Chem. Immunol. 51, 33-64.

Eastgate, J., Moreb, J.,Nick, H.S., Suzuki, K., Taniguchi, N., and Zucali, J.R. (1993) A role for manganese superoxide dismutase in radioprotection of hemotopoietic stem cells by interleukin-1. Blood 81, 639-646.

Elgin, S.C. (1988) The formation and function of DNase I hypersensitive sites in the process of gene activation. J. Biol. Chem. 263, 19259-19262.

Ephrussi, A., Church, G.M., Tonegawa, S., and Gilbert, W. (1985). B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science 227, 134-140.

Faber, P.W. , Van, Rooij, H.C., Schipper, H.J., Brinkmann, A.O., and Trapman, J. (1993). Two different, overlapping pathways of transcription initiation are active on the TATA-less human androgen receptor promoter. The role of SpI. J. Biol. Chem. 268, 9296-9301.

Fan, C.-M. and Maniatis, T. (1991). Generation of p50 subunit of NF-kB by processing of p105 through an ATP-dependent pathway. Nature 354, 395-397.

Feinberg, A.P. and Volgelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6-13.

Felsenfeld, G. and McGhee, J.D. (1986). Structure of the 30 nm chromatin fibre. Cell 44, 375-377.

Fenton, M.J., Clark, B.D., Collins, K.L., Webb, A.C., Rich, A., and Auron, P.E. (1987). Transcriptional regulation of the human prointerleukin 1 beta gene. J. Immunol. 138, 3972-3979.

Fibbe, W.E., Schaafsma, M.R., Falkenburg, J.H.F., and Willemze, R. (1989). The biological activities of interleukin-1. Blut. 59, 147-156.

Fischer, H.D., Dodgson, J.B., Hughes, S., and Engel, J.D. (1984). An unusual 5' splice sequence is efficiently utilized in vivo. Proc. Natl. Acad. Sci. U.S.A. 81, 2733-2737.

Freeman, B.A. and Crapo, J.D. (1982). Free radicals and tissue injury. Lab. Invest. 47, 412-426.

Freeman, B.A., Mason, R.J., Williams, M.C., and Crapo, J.D. (1986). Antioxidant enzyme activity in alveolar type II cells after exposure of rats to hyperoxia. Exp. Lung. Res. 10, 203-222.

Fridovich, I. (1974). Superoxide dismutases. Adv. Enzymol. 41, 36-97.

Fridovich, I. (1989). Superoxide dismutases. J. Bio. Chem. 264, 7761-7764.

Fritton, H.P., Igo-Kemeones, T., Nowock, J., Strech-Jurk, U., Theisen, M., and Sippel, A.E. (1984). Alternative sets of DNase I-hypersensitive sites characterize the various functional states of the chicken lysozyme gene. Nature 311, 163-165.

Galas, D.J. and Schmitz, A. (1978). DNAse footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. 5, 3157-3170.

Ghosh, S., Latimer, R.D., Gary, B.M., Harwood, R.J., and Oduro, A. (1993). Endotoxin-induced organ injury. Crit. Care Med. 21, s19-s24.

Goeddel, D.V., Aggarwal, B.B., Gray, P.W.; Leung, D.W., Nedwin, G.E.. Palladino, M.A., Patton, J.S., Pennica, D., Shepard, H.M., and Sugarman, B.J. (1986). Tumor necrosis factors: gene structure and biological activities. Cold Spring Harb. Symp. Quant. Biol. 51 Pt 1, 597-609.

Goodwin, R.G., Anderson, D., Jerzy, R., Davis, T., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Smith, C.A. (1991). Molecular cloning and expression of the type 1 and type 2 murine receptors for tumor necrosis factor. Mol. Cell. Biol. 11, 3020-3026.

Grimm, S. and Baeuerle, P.A. (1993). The inducible transcription factor NF-kB: structure-function relationship of its protein subunits. Biochem. J. 290, 297-308.

Gross, D.S. (1988). Nuclease hypersensitive sites in chromatin. Ann. Rev. Biochem. 57, 159-197.

Grosveld, F., van Assendelft, G.B., Greaves, D.R., and Kollias, B. (1987). Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell 51, 975-985.

Groudine, M., Peretz, M., and Weintraub, H. (1981). Transcriptional regulation of hemoglobin switching in chicken embroys. Mol. Cell. Biol. 1, 281-288.

Gullberg, U., Lantz, M., Lindvall, L., Olsson, I., and Himmler, A. (1992). Involvement of an Asn/Val cleavage site in the production of a soluble form of a human tumor necrosis factor (TNF) receptor. Site-directed mutagenesis of a putative cleavage site in the p55 TNF receptor chain. Eur. J. Cell. Biol. 58, 307-412.

Hahn, S. (1993). Efficiency in activation. Nature 363, 672-673.

Hass, M.A. and Massaro, D. (1987). Developmental regulation of rat lung Cu, Zn-superoxide dismutase. Biochem. 246, 697-703.

Hecht, A., Berkenstam, A., Stromstedt, A.E., Gustafsson, J.A., and Sippel, A.E. (1988). A progesterone responsive element maps to the far upstream steroid dependent DNase hypersensitive site of chicken lysozyme chromatin. EMBO J. 7, 2083-2073.

Heller, R.A., Song, K., Fan, N., and Chang, D.J. (1992). The p70 tumor necrosis factor receptor mediates cytotoxicity. Cell 70, 47-56.

Hjalmarsson, K., Marklund, S., Engstrom, A., and Edlund, T. (1987). Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase. Proc. Natl. Acad. Sci. U.S.A. 84, 6340-6344.

Ho, Y.S. and Crapo, J.D. (1987). cDNA and deduced amino acid sequence of rat copper-zinc-containing superoxide dismutase. Nucleic Acids Res. 15, 6746.

Ho, Y.S., Howard, A.J., and Crapo, J.D. (1991). Molecular structure of a functional rat gene for manganese-containing superoxide dismutase. Am. J. Respir. Cell Mol. Biol. 4, 278-286.

Hohmann, H.P., Remy, R., Poschl, B., and Loon, A.P.G.M. (1990). Tumor necrosis factors-α and-β bind to the same two types of tumor necrosis factor receptors and maximally activate the transcription factor NF-kB at low receptor occupancy and within minutes after receptor binding. J. Biol. Chem. 265, 15183-15188.

Hurt, J., Hsu, J.L., Dougall,W.C., Visner, G.A., Burr, I.M., and Nick, H.S. (1992). Multiple mRNA species generated by alternate polyadenylation from the rat manganese superoxide dismutase gene. Nucleic Acids Res. 20, 2985-2990.

Johnson, P.F. and McKnight, S.L. (1989). Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. 58, 799-839.

Jones, G.R. (1986) Free radicals in immunological killing: the case of tumor necrotising factor (TNF). Med. Hypotheses 21, 267-271.

Kikuchi, K., Bichell, D.P., and Rotwein, P. (1992). Chromatin changes accompany the developmental activation of insulin-like growth factor I gene transcription. J. Biol. Chem. 267, 21505-21511.

Kong, X.J., Lee, S.L., Lanzillo, J.J., and Fanburg, B.L. (1993). Cu, Zn superoxide dismutase in vascular cells: changes during cell cycling and exposure to hyperoxia. Am. J. Physiol. 264, L365-375.

Krall, J., Bagley, A.C., Mullenbach, G.T., Hallewell, R.A., and Lynch, R.E. (1988). Superoxide mediates the toxicity of paraquat for cultured mammalian cells. J. Bio. Chem. 263, 1910-1914.

Kushner, I., Ganapathi, M., and Schultz, D. (1989). The acute phase response is mediated by heterogeneous mechanisms. Annals N. Y. Acad. Sci. 557, 19-30.

Langston, A.W. and Gudas, L.J. (1992). Identification of a retinoic acid responsive enhancer 3' of the murine homeobox gene Hox-1.6. Mech. Dev., 38, 217-227.

Lantz, M., Gullberg, U., Nilsson, E., and Olsson, I. (1990). Characterization in vitro of a human tumor necrosis factor-binding protein. A soluble form of a tumor necrosis factor receptor. J. Clin. Invest. 86, 1396-1402.

Larrick, J.W. and Kunkel, S.L. (1988). The role of tumor necrosis factor and interleukin 1 in the immunoinflammatory response. Pharmaceutical Res. 5, 129-139.

Le, J. and Vilcek, J. (1987). Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab. Invest. 56, 234-248.

Lee, W., Mitchell, P., and Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49, 741-752.

Lei, M. and Morrison, D.C. (1988). Specific endotoxic lipopolysaccharide-binding proteins on murine splenocytes. J. Immunol. 141, 1006-1011.

Levanon, D., Lieman-Hurwitz, J., Dafni, N., Wigderson, M., Sherman, L., Bernstein, Y., Laver-Rudich, X., Daniger, E., Sein, O., and Groner, Y. (1985). Architecture and anatomy of the chromosomal locus in human chromosome 21 encoding the CU/Zn superoxide dismutase. EMBO J. 4, 77-84.

Liska, K.J., Robinson, V.R., and Bornstein, P. (1992). Elements in the first intron of the alpha 1 (I) collagen gene interact with SpI to regulate gene expression. Gene. Expr. 2, 379-389.

Lomedico, P.T., Gubler, U., Hellmann, C.P., Dukovich, M., Giri, J.G., Pan, Y.C., Collier, K., Semionow, R., Chua, A.O., and Mizel, S.B. (1984). Cloning and expression of murine interleukin-1 cDNA in Escherichia coli. Nature 312, 458-62.

Lowenthal, J.W., Ballard, D.W., Bohnlein, E., and Greene, W.C. (1989). Tumor necrosis factor alpha induces proteins that bind specifically to kappa B-like enhancer elements and regulate interleukin 2 receptor alpha-chain gene expression in primary human T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 86, 2331-2335.

Lund, S.D., Gallagher, P.M., Wang, B., Porter, S.C., and Ganschow, R. (1991). Androgen responsiveness of the murine B-glucuronidase gene is associated with nuclease hypersensitivity, protein binding, and haplotype-specific sequence diversity within intron 9. Mol. Cell. Biol. 11, 5426-5434.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). In T. Maniatis, E.F. Fritsch, and J. Sambrook (eds.). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Lavoratory, Cold Spring Harbor, N.Y.

Marklund, S.L. (1984). Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. Biochem. J. 222, 649-655.

Matsubara, T. and Ziff, M. (1986). Increased superoxide anion release from human endothelial cells in response to cytokines. J. Immunol. 137, 3295-3298.

McGhee, J.D., Wood, W.I., Dolan, M., Engel, J.D., and Felsenfeld, G. (1981). A 200 base pair region at the 5' end of the chicken adult beta-globin gene is accessible to nuclease digestion. Cell 27, 45-55.

Mitchell, P.J. and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245, 371-378.

Miller, M.R., Castellot, J.F.Jr., and Pardee, A.B. (1978). A permeable animal cell preparation for studying macromolecular synthesis. DNA synthesis and the role of deoxyribonucleotides in S phase initiation. Biochem. 17, 1073-1080.

Mizel, S.B., Kilian, P.L., Lewis, J.C., Paganelli, K.A., and Chizzonite, R.A. (1987). The interleukin 1 receptor. Dynamics of interleukin 1 binding and internalization in T cells and fibroblasts. J. Immunol. 138, 2906-2912.

Molloy, R.G., Mannick, J.A., and Rodrick, M.L. (1993). Cytokines, sepsis and immunomodulation. Br. J. Surg. 80, 289-297.

Mosley, B., Urdal, D.L., Prickett, K.S., Larsen, A., Cosman, D., Conlon, P.J., Gillis, S., and Dower, S.K. (1987). The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. J. Biol. Chem. 262, 2941-2944.

Muegge, K., Williams, T.M., Kant, J., Karin, M., Chiu, R., Schmidt, A., Siebenlist, U., Young, H.A., and Durum, S.K. (1989). Interleukin-1 costimulatory activity on the interleukin-2 promoter via AP-1. Science 246, 249-251.

Nedwin, G.E., Naylor, S.L., Sakaguchi, A.Y., Smith, D., Jarrett-Nedwin, J., Pennica, D., Goeddel, D.V., and Gray, P.W. (1985). Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. Nucleic Acids Res. 13, 6361-6373.

Neta, R., Douches, S., and Oppenheim, J.J. (1986). Interleukin 1 is a radioprotector. J. Immunol. 136, 2483-2485.

Niitsu, Y., Watanabe, N., Sone, H., Neda, H., Yamauchi, N., and Urushizaki, I. (1985). Mechanism of the cytotoxic effect of tumor necrosis factor. Jpn. J. Cancer Res. 76, 1193-1197.

Oberley, L.W., Kasemset St. Clair, P., Autor, A.P., and Oberley, T.D. (1987). Increase in manganese superoxide dismutase activity in the mouse heart after X-irradiation. Arch. Biochem. Biophys. 254, 69-80.

Pauli, U., Chrysogelos, S., Stein, J., and Stein., G. (1988). Native genomic blotting: high-resolution mapping of DNase-I-hypersensitive sites and protein-DNA interactions. Proc. Natl. Acad. Sci. U.S.A. 86, 16-20.

Pennica, D., Nedwin, G.E., Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B., and Goeddel, D.V. (1984). Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature. 312, 724-729.

Perlmutter, D.H., Goldberger, G., Dinarello, C.A., Mizel, S.B., and Colten, H.R. (1986). Regulation of class III major histocompatibility complex gene products by interleukin-1. Science 232, 850-852.

Pfeifer, G.P. and Riggs, A.D.(1991). Chromatin differences between active and inactive X chromosomes revealed by genomic footprinting of permeabilized cells using DNase I and ligation-mediated PCR. Genes Develop, 1102-1113.

Philipsen, S., Pruzina, S., and Grosvel, F. (1993). The minimal requirements for activity in transgenic mice of hypersensitive site 3 of the β globin locus control region. EMBO J. 12, 1077-1085.

Pugh, B.F. and Tjian, R. (1992). Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. J. Bio. Chem. 67, 679-682.

Rattner, J.B. and Lin, C.C. (1985). Radial loops and helical coils coexist in metaphase chromosomes. Cell 42, 291-296.

Richmond, T.J., Finch, J.T., Rushton, B., RHodes, D. and Klug, A. (1984). Structure of the nucleosome core particle at 7 Å resolution. Nature 311, 532-537.

Rietschel, E.H. and Brade, H. (Aug. 1992). Bacterial endotoxin. Scientific American 54-61.

Rigby, P.W.J. (1993). Three in one and one in three: it all depends on TBP. Cell 72, 7-10.

Rosen, D.R., Siddique, R., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., and Deng, H.X. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59-62.

Sachs, A.B. (1993). Messenger RNA degradation in eukaryotes. Cell 74, 413-421.

Saito, H. (1989). Decrease in Cu-Zn-superoxide dismutase mRNA level during differentiation of human monocytic and promyelotic leukemia cells. FEBS Lett. 249, 253-256.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467.

Sargent, T.D., Wu, J.-R.,. Sala-Trepat, J.M., Wallace, R.B., Reyes, A.A., and Bonner, J. (1979). The rat serum albumin gene: analysis of cloned sequences. Proc. Natl. Acad. Sci. U.S.A. 76, 3256-3260.

Schulze-Oshthoff, K., Bakker, A.C., Banhaesebroeck, B., Beyaert, R., Jacob, W.A., and Fiers, W. (1992). Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. J. Biol. Chem. 267, 5317-5323.

Schumann, R.R., Leong, S.R., Glaggs, G.W., Gray, P.W., Wright, S.D., Mathison, J.C., Tobias, P.S., and Ulevitch, R.J. (1990). Structure and function of lipopolysaccharide binding protein. Science 249, 1429-1431.

Sen, R. and Baltimore, D. (1986). Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell 47, 921-928.

Shapiro, M. B. and Senapathy, P. (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. 15, 7155-7175.

Shiki, Y., Meyrick, B.O., Brigham, K.L., and Burr, I.M. (1987). Endotoxin increased superoxide dismutase in cultured pulmonary endothelial cells. Am. J. Physiol. 252, c436-440.

Sims, J.E., March, C.J., Cosman, D., Widmer, M.B., MacDonald, H.R., McMahan, C.J., Grubin, C.E., Wignall, J.M., Jackson, J.L.. and Call, S.M. (1988). cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. Science 241, 585-589.

Sinclair, A.J., Barnett, A.H., and Lunce, J. (1990). Free radicals and antioxidant systems in health and disease. Br. J. Hospital Med. 43, 334-340.

Slot. J., Geuze, H., Freeman, B., and Crapo, (1986). Intracellular localization of the copper-zinc and manganese superoxide dimutases in rat liver parenchymal cells. Lab. Invest. 55, 363-371.

Steffens, G.J., Michelson, A.M., Puget, K., and Flohe, L. (1986). The amino-acid sequence of rat Cu-Zn superoxide dismutase. Biol. Chem. Hoppe Seyler 367, 1017-1024.

Strieter, R.M., Kunkel, S.L., Showell, H.J., Remick, D.G., Phan, S.H., Ward, P.A.,and Marks, R.M. (1989). Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta. Science 243, 1467-1469.

Svingen, B.A., O'Neal, F.O.,and Aust, S.D. (1978). The role of superoxide and singlet oxygen in lipid peroxidation. Photochem. Photobiol. 28, 803-809.

Tartaglia, L.A. and Goeddel, D.V. (1992). Two TNF receptors. Immunol. Today 13, 151-153.

Tartaglia, L.A., Rothe, M., Hu, Y.-F.,and Goeddel, D.V. (1993). Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. Cell 73, 213-216.

Tobias, P.S., Mathison, J.C.,and Ulevitch, R.J. (1988). A family of lipopolysaccharide binding proteins involved in responses to Gram-negative sepsis. J. Biol. Chem. 263, 13479-13481.

Tsujimoto, M., Yip, Y.K., and Vilcek, J. (1985). Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. Proc. Natl. Acad. Sci. U.S.A. 82, 7626-7630.

Tuan, D., Soloman, D., Li, O., and London, I.M. (1985). The β-like globin gene domain in human erythroid cells. Proc. Natl. Acad. Sci. U.S.A. 82, 6384-6388.

Turrens, J.F. and Boveris, A. (1980). Generation of superoxide anion by NADH dehydrogenase of bovine heart mitochondria. Biochem. J. 191, 421.

Urban, M.B., Schreck, R., and Baeuerle, P.A. (1991). NF-kB contacts DNA by a heterodimer of the p50 and p65 subunit. EMBO J. 10, 1817-1825.

Visner, G.A., Dougall, W.C., Wilson, J.M., Burr, I.A., and Nick, H.S. (1989). Regulation of manganese superoxide dismutase by lipopolysaccharide, interleukin-1, and tumor necrosis factor. J. Biol. Chem. 265, 2856-2864.

Ward, P.A., Warren, J.S., and Johnson, K.J. (1988). Oxygen radicals, inflammation, and tissue injury. Free Radic. Biol. Med. 5: 403-408.

Weisinger, R.A. and Fridovich, I. (1973). Superoxide dismutase: organelle specificity. J. Biol. Chem. 248, 3582-3592.

Weiss, E., Ruhlmann, C., and Oudet, P. (1986). Transcriptionally active SV40 minichromosomes are restriction enzyme sensitive and contain a nucleosome-free origin region. Nucleic Acids Res. 14, 2045-2058.

Welbourn, C.R.B. and Young, Y. (1992). Endotoxin, septic shock and acute lung injury: neutrophils, macrophages and inflammatory mediators. Br. J. Surg. 79, 998-1003.

Wiley, S.R., Kraus, R.J., and Bertz, J.E. (1992). Functional binding of the "TATA" box binding component of transcription factor TFIID to the -30 region of TATA-less promoters. Proc. Natl. Acad. Sci. U.S.A. 89, 5814-5818.

Wispe, J.R., Warner, B.B., Clark, J.C., Dey, C.R., Neuman, J., Glasser, S.W., Crapo, J.D., Chang, L.Y., and Whitsett, J.A. (1992). Human Mn-superoxide dismutase in pulmonary epithelial cells of transgenic mice confers protection from oxygen injury. J. Biol. Chem. 267, 23937-23941.

Wong, G.H.W. and Goeddel, D.V. (1988). Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. Science 242, 941-944.

Wong, G., Elwell, J., Oberley, L., and Goeddel, D. (1989). Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity by tumor necrosis factor. Cell 58, 923-931.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., and Mathison, J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431-1433.

Wu, C., Bingham, P.M., Livak, K.J., Holmgren, R., and Elgin, S.C. (1979). The chromatin structure of specific genes: I. evidence fo higher order domains of defined DNA sequence. Cell 16, 797-806.

Wu, C. (1980). The 5' Ends of Drosophila heat shock genes in chromatin are hypersensitive to DNase I. Nature 286, 854-860.

BIOGRAPHICAL SKETCH

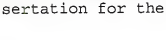
Jan-ling Hsu was born in Taiwan on June 30, 1961. She attended National Taiwan University, Taiwan, in 1979, and received her Bachelor of Science degree in pharmacy in 1983 and Master of Science degree in biochemistry in 1985. In August 1987, she came to the U.S.A. to pursue a doctoral degree in the Department of Biochemistry and Molecular Biology at the University of Florida. Under the supervision of Dr. Harry S. Nick she received her Doctor of Philosophy degree in biochemistry and molecullar biology in December, 1993.

She was married to Fongyi Shew in May, 1990, and their daughter, Victoria Yu-Tsu Shew, was born in February, 1993.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

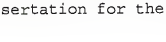
Harry S. Nick, Chairman
Associate Professor of
Biochemistry and Molecular
Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



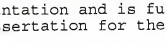
Richard P. Boyce
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Brian D. Cain
Assistant Professor of
Biochemistry and Molecular
Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Robert J. Ferl
Professor of Plant Molecular
and Cellular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Susan C. Frost
Associate Professor of
Biochemsitry and Molecular
Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1993

Mohan K Raizada
Dean, College of Medicine

Karen A. Holbrook
Dean, Graduate School